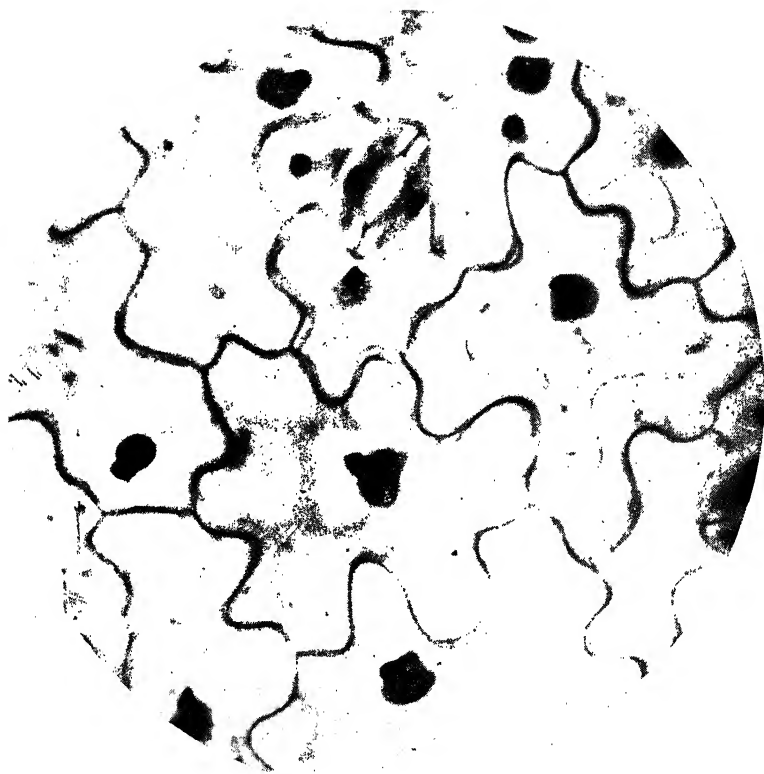


• A NEW SERIES OF PLANT SCIENCE BOOKS •

edited by Frans Verdoorn

Volume XIII

PLANT VIRUSES
and
VIRUS DISEASES



EPIDERMAL STRIP OF *Solanum nodiflorum* INFECTED WITH TOMATO AUCUBA MOSAIC VIRUS. FIXED IN CARNOY'S FLUID AND STAINED WITH FEULGEN'S REAGENT AND METHYLENE BLUE. THE NUCLEI HAVE REACTED WITH THE FEULGEN, WHEREAS THE LARGER, VACUOLATE X-BODIES HAVE BEEN STAINED ONLY WITH THE METHYLENE BLUE. $\times 650$ (After J. Henderson Smith, ANN. APPL. BIOL. 17:213, 1930).

PLANT VIRUSES *and* VIRUS DISEASES

BY

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—SECOND ENTIRELY REVISED EDITION—



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PREFACE to SECOND EDITION

With the invasion of the Netherlands the type of the first edition was lost, so that a second printing could not be made and the edition was out of print within a year of publication. Despite the war, work on viruses and virus diseases has made considerable progress in the last three years, and the need to set up new type has been made the opportunity for bringing the text up to date and for adding new illustrations. An attempt has been made to include all new work, but the difficulties of getting literature in present circumstances almost inevitably means that some has been missed. More than half the chapters have been rewritten and all have been modified to some extent. The most extensive alterations have been needed in chapters dealing with the properties of viruses in vitro and with their relationships to their insect vectors, for in these aspects of the subject our knowledge has grown most rapidly. Some of the many controversies of 1939 have been resolved. In particular, few biologists now question that the specific proteins isolated from infected plants represent the viruses themselves and all workers are agreed that tobacco mosaic virus is a nucleoprotein, so that there has been less need to emphasise the evidence for these views in this edition. Unfortunately we still know little of the behaviour of viruses in their natural environment, that is, within the host plants: this aspect now calls for much increased attention and can be expected to provide the next major advances in the subject.

I am deeply indebted to Dr. and Mrs. VERDOORN for the great care they have taken over the preparation of this edition and for kindly correcting the proofs and making the author index.

HARPENDEN

Autumn 1942

PREFACE to FIRST EDITION

At the present time the study of plant viruses is in a transitional stage. Until recently, work on viruses has been left largely in the hands of pathologists, for, although the exact position of the subject has always been a little uncertain, viruses undoubtedly caused diseases and were therefore conveniently described with the pathogenic bacteria and fungi. In the past few years, however, the subject has received increasing attention from workers in other fields. Chemists, crystallographers, entomologists, geneticists, serologists, physicists, and others are becoming attracted by these intriguing pathogens. They are bringing fresh techniques to the subject and are greatly increasing our knowledge. One result of this is that publications on viruses are becoming even more widely scattered, in apparently unrelated journals, than before. Another is that the necessity for separating viruses from other recognised pathogens is becoming more obvious, and their adoption by protein chemists seems only a matter of time. This is not meant to be a text-book of virus diseases. Detailed descriptions of symptoms and host-ranges are not given, but all other aspects of the subject are treated. It is an attempt to describe and cor-

relate the advances that have been made recently in the study of plant viruses. Many of the points described are still controversial, but I have tried to distinguish between facts and the deductions made from them.

HARPENDEN

July 1939

Acknowledgments

*It is a pleasure to express my gratitude to my colleagues for their generous help and advice, especially to Mr. N. W. PIRIE for his invaluable constructive criticisms. I also wish to thank authors and journals for permission to reproduce illustrations. The sources of these are acknowledged individually in the text. Acknowledgment also is made to "The Annals of Applied Biology" for the loan of a number of blocks. — The vignette on page xiv has been reproduced from HERMANN SCHACHT'S Bericht an das Königliche Landes-Oekonomie-Kollegium über die Kartoffelpflanzen und deren Krankheiten (Berlin 1856, Taf. VI, 11) and is probably the first illustration of potato curl. The vignettes on pages 284 and 294 show the oldest known plant virus disease, tulip mosaic or break, after CLUSIUS' wood cuts of variegated early tulips (*Rariorum aliquot stirpium, per Pannoniæ etc., Antverpiæ 1583*; cf. MCKAY and WARNER, *Nat. Hort. Mag.*, July 1933, p. 187).*

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INTRODUCTORY SURVEY

Introduction:—The name virus disease was in current use in France during the latter half of last century to describe those diseases, typified by small pox, which confer an immunity from subsequent attacks on men and animals who have once suffered and recovered. Although many diseases with this characteristic property are still recognised as virus diseases, others are not, for it is no longer used as a criterion. Indeed, there is now no such single positive test for distinguishing virus diseases, those described under this name being grouped together because they have other distinguishing characters in common.

An organism is diseased when its condition departs in any appreciable manner from the normal. Abnormal conditions can, of course, result from many different causes, but they divide sharply into infectious and non-infectious diseases, depending on whether or not the causes can be transmitted to healthy organisms and the abnormal conditions reproduced in them. Again, for the purposes of this book, the infectious diseases can conveniently be divided into two on the basis of the visibility of their causes. Firstly, there are those caused by obvious parasites, typified by insect, fungal and bacterial diseases, and, secondly, those for which no visible causes have yet been satisfactorily demonstrated. The name virus disease is now usually restricted to infectious diseases of the second type.

The intensive study of viruses and virus diseases is a relatively recent development. Their existence was not demonstrated until 1892 and little attention was paid to them at first. Nevertheless the subject already ranks as one of the most important parts of pathology, and is receiving increasing attention from workers in other branches of science. Its economic importance is difficult to exaggerate. Except for the bacteria, no lower plants have been discovered with virus diseases, but few other organisms seem to escape attack and new diseases of this type are continually being described. Exact information on the losses resulting from virus diseases cannot easily be obtained, but a number of writers have stated that they at least equal those caused by the visible bacteria. Two reliable figures can be given for Great Britain: on an average the Ministry of Agriculture pays two hundred thousand pounds a year in compensation to farmers for animals slaughtered in preventing foot-and-mouth disease from becoming endemic as it is in many other European countries, and there are few serious complaints against this policy. Also, farmers in England annually spend around seven hundred thousand pounds for new potato seed from Scotland and Ireland to replace their own stocks that have become virus diseased. Numerous other figures have been

published, but as most of these are merely estimates, listing the names of a few of the more widely known virus diseases will probably better indicate their economic importance than attempting to show it in largely fictitious money values.

Small pox, measles, mumps, herpes, yellow fever, influenza and infantile paralysis are only a few of the virus diseases of man. Among those affecting animals are swine fever, louping ill, horse sickness, encephalomyelitis, rabies, distemper and foot-and-mouth. Birds suffer from many kinds of pox, plague, tumours, and psittacosis, and insects from sacbrood and polyhedral diseases. Bacteria are destroyed by the bacteriophages, and plants are attacked by the numerous degeneration diseases and infectious chloroses that form the subject of this book.

At first sight the diseases listed form an extremely heterogeneous collection, for their symptoms could hardly be more varied. The justification for grouping these diverse abnormal conditions together under the name virus diseases lies in the peculiar properties of their causes. No visible parasite has yet been isolated capable of producing any of them. In this respect they resemble the deficiency diseases and those caused by toxins, but they are separated sharply from these in being highly infectious. Their causes, *i.e.*, the viruses, are obviously pathogens which multiply in suitable hosts, for the diseases can be reproduced indefinitely in series by transmission from diseased to healthy organisms. Although the viruses multiply so readily in susceptible living tissues, none has yet been cultivated *in vitro*, or when freed from host materials has been proved to have any detectable metabolism.

The fact that they cannot be seen even under the best microscopes suggests that viruses are much smaller than other recognised pathogens. Their small size is further emphasised by the ability of many to pass through filters with pores sufficiently small to stop all ordinary bacteria. The existence of viruses was first demonstrated by the discovery that bacteriologically sterile filtrates could be infectious. Because of this they have often been called "filterable-viruses", and passage through such filters has been used as the decisive test for a virus. However, this criterion, which was always less used in plant than in animal pathology, has now largely been abandoned for it has been found to be open to many objections. It is known that many factors other than the relative sizes of particles and pores can determine whether or not the particles will pass through the pores. An unsuitable suspension medium or a too small virus content, absorption on to either the filter or on to other constituents of the preparation, may all play important parts in preventing a virus from passing through a filter. The fact that a pathogen is not filterable, therefore, cannot be taken as proof that it is not a virus. With some plant diseases included in the virus group it is even impossible to determine whether the causes are filterable. The only known method of transmitting these is by grafting portions of diseased plants to healthy ones, so that there is at present no method whereby the filtrates could be tested for the presence of virus. Nor can passage through a filter be taken as proof that a pathogen is a virus. Filterable forms

of bacteria have been described, and some other visible organisms, such as the flexible spirochaetes, can also pass through filters usually regarded as impervious to bacteria.

The distinguishing features of viruses, therefore, can briefly be summarised as ability to cause disease, inability to multiply *in vitro* with ordinary cultural methods, and small size relative to other types of recognised pathogens. Since filterability has been found to be unsuitable as a criterion of small size, invisibility can probably be better used in any attempts to define viruses. Visibility under the microscope depends on the refractive index of the particles examined, the numerical aperture of the objective and the wavelength of the light. Provided that their refractive index differs sufficiently from that of the mounting medium, particles of 200 m μ diameter can be seen by ordinary transmitted light by means of their diffraction images. The shape of particles of this size cannot be demonstrated, nor can the combined image of two adjacent particles be resolved into its separate parts. For the particles to be clearly resolvable with the best objectives by ordinary microscopical methods, their dimensions must exceed 250 m μ . With the knowledge that plant viruses are not resolvable by ordinary microscopical methods, therefore, it is possible to define a virus as an obligately parasitic pathogen with at least one dimension of less than 200 m μ .

Such a definition will almost certainly be modified by future work, but it will serve as a working hypothesis for the present. It is probable that viruses will be defined eventually much more accurately on the basis of either their chemical constitution or activities. A good deal is already known about the chemical composition of some plant viruses, but too few have yet been studied to know how generally this knowledge can be applied. The suggested definition covers the facts as known at present, but it is obviously artificial. There is no obvious reason why the resolving power of the microscope should have any particular biological significance. There are many organisms, both microscopic and macroscopic, which appear to be obligate parasites. Of plant pathogens, no rust or powdery mildew has yet been cultivated on artificial media; nor has *Plasmodiophora brassicae* or *Synchytrium endobioticum*. Obligate parasitism, therefore, is not solely a property of small agents. Whether it is a necessary sequel to small size cannot yet be stated. More fungi and bacteria are saprophytes than are parasites, and it is possible that saprophytic agents similar to the parasitic viruses exist, but the difficulties of recognising them would be enormous. However, BARNARD (1935) has already produced some microscopical evidence for the existence of what he terms "saprophytic viruses". Using ultra-violet light and a microscope of high resolving power, he has photographed bodies of the size of many viruses, and these readily multiply in horse- and rabbit-serum. Furthermore, it is possible that there are parasitic agents that cause no symptoms in any infected organisms, but it is difficult to see how these could at present be differentiated from the normal constituents of organisms. Thus until such saprophytes or hypothetical parasites can be shown to resemble the pathogenic viruses in properties more fundamental than size, it would seem preferable to restrict the

name virus to agents fulfilling all three requirements of the suggested definition.

Historical:—Although the number of recognised virus diseases increases steadily, and many workers are convinced that they are much more common than they were a generation or more ago, there is no reason to consider them to be afflictions peculiar to modern times. Smallpox and some other diseases of man have undoubtedly existed for centuries, and ZINSSER, in his interesting book *Rats, Lice and History*, shows the important effect some of these, in epidemic form, have had on history in the past.

There are only a few records of the many plant maladies now recognised as virus diseases until comparatively recent years. Breaking of the flower colour in Tulips, however, is mentioned as early as 1576. The potato, one of the greatest sufferers, is known to have been so severely attacked by 1775 that farmers in parts of Europe had to give up the cultivation of this plant, and prizes were offered to those who would find the cause of and remedy for the destructive disease of such a useful plant. Numerous papers were published around this period. The majority stress the degenerate state of affected plants and the condition became widely known as “degeneration”, “running-out” or “senility”. It was early noticed that new varieties were less affected than old ones, an observation resulting in the generally accepted view that the condition was a result of the “unnatural” continued propagation by asexual methods. Little experimental work was done at this time, but ANDERSON (1792) in an essay written in 1778 suggested many things that recent work has confirmed. He noticed that affected plants always gave rise to diseased progeny, and that plants grown in the south of the British Isles suffered more than those in the north. He compared the disease with smallpox, suggested that it was infectious and that it could be greatly reduced by removing all diseased plants as soon as they were fairly above ground. It seems likely that potato viruses were actually introduced into Europe at about this time, for most writers are agreed that the degeneration was unknown before 1770.

About 1868 the variegated *Abutilon* was popular in Europe as an ornamental plant, and it was found that the variegation could be transmitted to the normal green *Abutilon* by grafting. SMITH in 1891 and 1894 described peach yellows and peach rosette, and found that healthy trees contracted the diseases when grafted with buds taken from diseased ones.

The mosaic of tobacco was recognised by SWIETEN in 1857, but was first named and adequately described by MAYER in 1886. MAYER's work may reasonably be regarded as the beginning of modern studies on virus diseases. He showed that the cause could be transmitted by mechanical inoculation methods, for when he stuck capillary tubes filled with sap from affected plants into healthy ones these became diseased.

During the nineteenth century many of the papers on the “curl” of potatoes contain statements to the effect that no fungus or bacterium could be found responsible for the condition. PASTEUR, also,

could find no organism causing rabies, and he appears to have been the first to suggest the possibility that there might exist pathogens too small to be seen. But it was not until 1892 that proof of their existence was obtained. IWANOWSKI, a Russian botanist, then showed that tobacco mosaic could be produced by inoculating plants with the juice from infected plants after it had been filtered through bacteria-proof filters. At the time, IWANOWSKI's results do not seem to have attracted the attention they warranted, but in 1898 they were independently confirmed by BEIJERINCK. In the same year, LOEFFLER and FROSCHE showed that the foot-and-mouth disease of cattle was also caused by a filter passer. Since then so many diseases have been described as virus diseases that it is impossible to attempt a review. At first filterability was used as a criterion, but later many diseases were attributed to viruses, because of their close similarity with other diseases shown to be caused by filter-passers, without passing this test.

The early work was concerned chiefly with symptomatology, the identification of new diseases, and studies on transmission. In 1900 REED and others showed that yellow fever was transmitted by a mosquito, and in 1901 the important part that insects are now known to play in the transmission of plant viruses was indicated by TAKAMI who succeeded in transmitting the "stunt" disease of rice by means of a leaf-hopper, *Nephotettix apicalis*. It is rather surprising in view of these important early discoveries that the possibility of their wide application was not realised, and that progress was at first so slow. It was not until 1913 that ORTON showed that the "degeneration" of the potato took more than one form. He then described three types of symptoms under the names leaf roll, mosaic and streak, but it was only in 1916 that QUANER showed leaf roll to be infectious and 1920 before the transmission of this disease by insects was indicated by OORTWIJN BOUTES.

The fact that plants could act as "carriers", that is to say, become infected but show no obvious abnormalities, was discovered in 1918. NISHIMURA then found that *Physalis Alkekengi* showed no symptoms when inoculated with tobacco mosaic virus, although sap from these plants caused typical mosaic when rubbed on to healthy tobacco plants. Naturally occurring carriers were found in potatoes by SCHULTZ (1925) and JOHNSON (1925). Indeed, one virus was found with such regularity in American potatoes, whether apparently healthy or obviously diseased, that it became known as the "healthy potato virus".

Some of the most common virus diseases, especially of the potato, are now known to be caused by simultaneous infection with two distinct viruses, the two together often producing symptoms quite distinct from those produced by either when present alone. The first disease shown to be caused by a virus-complex of this type was glass-house streak or "winter blight" of tomatoes. VANTERPOOL in 1926 found that tomatoes infected with either a potato mosaic virus or tobacco mosaic virus singly developed only a mottle, whereas when infected with both they developed typical streak.

The alteration of smallpox by continued passage through calves

and the attenuation or "fixing" of rabies by PASTEUR, clearly indicated that some animal viruses possessed powers of adaptation long before their existence as a distinct type of pathogen was appreciated. Suggestions that plant viruses might also exist in strains of different virulence, and that one strain might arise from another by a process presumably akin to mutation, are first found in the work of CARSONER (1925), JOHNSON (1926) and MCKINNEY (1926). Since then the majority of plant viruses have been found to exist in a multiplicity of strains. In general, the individual strains of one virus have closely similar physical and chemical properties, but differ in their virulence, the type of symptoms they cause and in their host range. The intensive study of the effect of mixing strains in infected plants has led to the discovery of the first fully established examples of acquired immunity in plants. In 1931 THUNG showed that if tobacco plants infected with tobacco mosaic virus were inoculated with sap from plants with "white mosaic" they failed to develop the symptoms of this disease. SALAMAN (1933) also found that plants infected with an avirulent strain of potato virus "X" produce no further symptoms when re-inoculated with virulent strains. This phenomenon has been so often confirmed, both with these two and with other viruses, that it now seems probable that plants infected with one strain of any virus become resistant to infection with other strains of that virus, although remaining susceptible to infection with other unrelated viruses.

Before leaving this brief historical survey of work on virus diseases, two other discoveries that have greatly affected the methods of studying these diseases deserve mention. The first is the recognition of local lesions, *i.e.*, visible lesions produced at the site of infection, and their application by HOLMES (1928, 1929) in quantitative work when he found that the number produced was approximately proportional to the concentration of infective sap. The second is the finding that many plant viruses are antigenic and when injected into animals produce specific antibodies, with which the viruses react in some observable way. The first attempt to apply serological methods was made by DVORAK in 1927, but the early work of PURDY BEALE (1928, 1929, 1931) showed their importance and indicated the wide applications they are now known to have.

Views on the nature of viruses: — In addition to the attention that viruses have received because of their economic importance, the uncertainty as to their nature has continually attracted great scientific interest. Their invisibility, coupled with their apparent inability to multiply *in vitro*, rendered the usual bacteriological methods of little value in investigating viruses. In spite of this, the most generally accepted view has been that viruses are essentially similar to small bacteria, for the early work consisting largely of transmission studies and symptomatology brought few results to conflict with this. Other theories, however, have often been put forward, especially by those working on the physical and chemical properties of tobacco mosaic virus, but until the last few years there has been little definite evidence to indicate whether the viruses more nearly resemble organisms

such as the small bacteria or molecules such as the larger known proteins.

BEIJERINCK (1898) was the first to suggest that viruses might differ essentially from bacteria in factors other than size. He attempted to express this difference by calling the cause of tobacco mosaic a "*contagium vivum fluidum*". This suggestion was made when he found that the virus not only passed through bacteria-proof filters but also diffused through agar plates. It is probable that BEIJERINCK wished to convey the impression of something capable of forming a stable solution, a view largely confirmed by recent work.

WOODS (1899, 1900) considered that tobacco mosaic resulted from an excessive accumulation of oxidising enzymes. He suggested that adverse growing conditions increase the enzyme activity, so changing chlorophyll into xanthophyll and producing the characteristic variegations. That the virus was an enzyme or toxic principle was again suggested by HUNGER (1905) and FREIBERG (1917), but ALLARD (1916) showed that WOODS' theory was incorrect, and on reviewing the evidence then available came to the conclusion that the most probable cause was a micro-organism.

Peculiar intracellular inclusions had been found accompanying some diseases of animals many years before these were recognised as caused by viruses. As early as 1841 HENDERSON described the inclusions of *molluscum contagiosum*, and in 1869 and 1894 those of fowl-pox and vaccinia were described by RIVOLTA and GUARNIERI respectively. That such inclusions were a result of virus infection was indicated by IWANOWSKI in 1903, when he found two kinds in plants suffering from tobacco mosaic. One was an amoeboid body, often in contact with the nucleus, and the other a flat crystalline plate. Since then numerous workers have pictured these inclusions, and the amoeboid bodies have been found accompanying many diseases other than tobacco mosaic.

Various interpretations have been placed on these inclusions. IWANOWSKI concluded that they could not be organisms responsible for the disease as they were too large to pass through filters. He suggested that they were either products of nuclear division or reaction products of the cell to the disease. Although he was unable to isolate any bacteria, he saw numerous short rods resembling bacteria in stained sections, and concluded that these were the cause of the disease. Most workers agreed that the crystalline plates were merely plant reaction products, but opinion on the nature of the amoeboid bodies was sharply divided. SMITH (1924), HOGGAN (1927), HENDERSON SMITH (1930), and CLINCH (1931) supported the view of IWANOWSKI, but KUNKEL (1921), MCWHORTER (1922), LYON (1910), GOLDSTEIN (1924) and LIKHTE (1929) considered that they were stages in the life cycle of an organism responsible for the disease.

In addition to the intracellular inclusions, various other "organisms" have been described from time to time as the causes of plant virus diseases. NELSON (1922) first attributed mosaic of the bean to a protozoan and then later (1932) to a small coccus. ECKERSON (1926) described small motile organisms in the form of small flagellates as the cause of tomato mosaic. v. BREHMER and

BARNER (1930) picture small oval bodies in potatoes and beet suffering from mosaic, which they named *Plasmodiophora solani* and considered responsible for the diseases. PAINE and BEWLEY (1919) stated that *Bacillus lathyri* produced "stripe" of tomato, but later BEWLEY (1931) suggested that a bacteriophage entering the plants with the bacteria was the direct cause and not the bacteria themselves. As all the organisms that have been described are obviously too large to be filterable, a life cycle has usually been postulated in which visible stages alternate with an invisible, filterable stage. None of these theories has been confirmed. At the present time there is no reason to believe that an organism has been seen which is responsible for any of the recognised plant virus diseases.

With the failure of microscopic and cultural methods to reveal conclusively any cause for virus diseases, physical and chemical methods used for investigating proteins and other substances with large molecules have been increasingly applied. Most of the early work was done with tobacco mosaic virus, and the great stability of this virus was soon recognised. It was also found that it could be precipitated with various protein precipitants and resuspended without losing activity. These findings led to suggestions that the behaviour of the virus was more analogous to that of chemical substances such as proteins (MULVANIA 1926) than to that of organisms. They also led to attempts to isolate the virus by chemical methods. VINSON and PETRE (1929, 1931) obtained reasonably active, colourless preparations by the use of lead acetate, and from its behaviour concluded that tobacco mosaic virus was a nitrogen-containing chemical substance. BARTON-WRIGHT and M'BAIN (1933) claimed to have isolated this virus in the form of nitrogen-free crystals, but CALDWELL (1934) showed that their crystals were largely phosphate, contaminated with small amounts of virus.

In 1935 much more highly purified and concentrated preparations of tobacco mosaic virus were made by STANLEY, who claimed to have isolated "a crystalline protein possessing the properties of tobacco mosaic virus". The fact that infected plants contain a specific protein has now been confirmed by several workers, and further work has greatly strengthened the claim that the protein is the virus itself, although it has necessitated considerable modifications of STANLEY's original views. For example, BAWDEN and PIRIE (1936, 1937*a*) have shown that the protein is a nucleoprotein and not a globulin, while BERNAL and FANKUCHEN (1937) state that STANLEY's needle-shaped "crystals" lack the three-dimensional regularity characteristic of true crystals. They are one of the forms of the liquid crystalline state and are more accurately described as fibres or paracrystals.

This type of work has now been successfully applied to plants with diseases other than tobacco mosaic. BAWDEN and PIRIE (1937*b*; 1938*a*; 1938*b*; 1939) have isolated specific nucleoproteins from plants infected separately with cucumber viruses 3 and 4, two strains of potato virus "X", tomato bushy stunt virus, potato virus "Y" and *Hyoscyamus* virus 3. PIRIE and others (1938) obtained crystalline and amorphous nucleoproteins from plants infected with tobacco ne-

crossis viruses, and STANLEY (1939) and ROSS (1941) isolated still other nucleoproteins from plants suffering from tobacco ringspot and alfalfa mosaic respectively.

Such proteins have not been found in healthy plants, but they have been isolated from many different species of infected plants. The particular protein isolated is determined solely by the identity of the infecting virus, and has the stability characteristic of that virus. The purified preparations are highly infective, from 10^{-8} to 10^{-10} gms usually being sufficient to give infection when rubbed over the leaves of susceptible plants. At the present time it seems most reasonable and probable that these nucleoproteins are the viruses themselves, and this view will be adopted in this book as a working hypothesis. Proof of this could be obtained only by a conclusive demonstration that the purified virus preparations contain only one type of particle but in practice it is impossible to get such proof of purity. A great variety of tests has been made on the carefully purified viruses without giving any suggestion of serious heterogeneity. None of the tests alone would be sufficient to conclude that the preparations are pure, but taken all together they provide an almost overwhelming mass of evidence favouring this conclusion. If the viruses are not the proteins whose properties are described in this book, there would seem to be only two possible alternatives. The first is that they resemble these proteins in every property yet studied. The second is that the viruses are present in the purified preparations as minute contaminants so firmly adsorbed on to the proteins that no methods now available can separate the two. Substances which for long have been regarded as pure have often been found to be mixtures, and it may be so with these virus preparations. By adopting either of the alternative views, however, the problem becomes unnecessarily complicated. The viruses once again become mysterious somethings, but with the added complication that in addition to multiplying themselves they also cause the production of these specific and unusual nucleoproteins. Although this must be recognised as a possibility, until some evidence can be found to suggest it, it is gratuitous to postulate the presence of two entities when one is sufficient to satisfy the data. Or, in the words of OCCAM (*ca.* 1330), *Essentia non sunt multiplicanda praeter necessitatem*.

Nomenclature:—The absence of any systematic basis for the nomenclature of viruses has led to considerable confusion and is responsible for many of the apparent contradictions in published work. The same virus has often received different names from different workers, thereby leading to a multiplicity of synonyms, and sometimes the same name has been given to distinct viruses. This has come about largely as a result of naming viruses on the basis of the symptoms they cause, without appreciating that the same symptoms are not produced under all conditions by the same virus or that similar symptoms may be caused by different viruses.

At first, little attempt was made to distinguish between cause and effect, the virus and the disease usually being referred to as the same thing. Later a distinction was made by adding the word virus to the

name of the disease to indicate the cause, for example, tobacco mosaic was stated to be caused by tobacco mosaic virus. With increasing knowledge it became obvious that plants could be attacked by more than one virus, and that similar symptoms could be caused by different viruses. Also, it was found that the same virus might infect a large number of plant species, inducing different symptoms in different plants. This showed that the system of naming on the basis of the host attacked and the symptoms produced was inadequate. Also, in plants such as the potato and tobacco, which are attacked by a number of viruses producing mottling or necrosis, it became increasingly difficult to find sufficiently distinct descriptive names for all the viruses recognised. In 1927, therefore, JOHNSON suggested that descriptive names should be applied only to the diseases, the virus merely taking its name from the host in which it was first discovered, together with a number to indicate the specific virus. Thus the causes of tobacco mosaic and cucumber mosaic became *Tobacco virus 1* and *Cucumber virus 1* respectively. SMITH (1931) separated two viruses from a diseased potato and called them potato viruses "X" and "Y" respectively. This practice has been followed with other potato viruses, a large number having now been named alphabetically.

At the International Botanical Conference at Cambridge in 1930 an International Committee on Virus Nomenclature was set up under the Chairmanship of Professor JAMES JOHNSON to consider the whole question. This committee reported to the Conference in Amsterdam in 1935 and proposed a scheme that was adopted in principle, but it has never been officially published. This was an elaboration of JOHNSON's original scheme, designed to cover names for strains as well as for separate viruses. In addition to the common name of the host followed by the term virus and a number, strains were indicated by a capital letter and sub-strains by a small letter. Thus the common cucumber virus became cucumber virus 1, the strain producing a yellow mottle 1B and the variants isolated from this by PRICE (1934) 1Ba, 1Bb and so-on. Working with potato virus "X", SALAMAN (1933) used a different method of indicating strains; a further letter was allotted to each strain and written as an index, the severe strain causing ringspot symptoms in tobacco becoming X^s and the mild one causing a faint mottle X^m.

Since 1935 new systems of nomenclature have been put forward with such frequency by individual workers that a state approaching chaos has been reached, for even well-established names have been replaced by complicated unknowns, and the name used by one virus worker may convey little or nothing to another.

SMITH (1937) introduced a modification of the scheme tentatively proposed by the International Committee, in which the Latin generic name of the host is used instead of the common English name. Thus tobacco mosaic and aucuba mosaic viruses become *Nicotiana virus 1* and *1c*, respectively, and potato virus "X" becomes *Solanum 1a*. SMITH added to the confusion by keeping some of the numbers for individual viruses that had been proposed in JOHNSON's scheme and altering others.

HOLMES (1939) introduced a binomial system of nomenclature, similar to that in use for plants and animals, with a trinomial for indicating strains. In this scheme ordinary tobacco mosaic virus becomes *Marmor Tabaci*, var. *vulgare*, aucuba mosaic virus *Marmor Tabaci*, var. *Aucuba*, cucumber mosaic virus *Marmor cucumeris* var. *vulgare* and the strain of potato virus "X" causing ringspot in tobacco *Marmor dubium* var. *annulus*.

Both SMITH and HOLMES claim that their systems of naming are based on a classification of viruses, but for reasons discussed in Chapter 14 it is clear that the systems of grouping adopted hardly warrant the term classification.

The limited use of numbers or letters worked reasonably well, but the extension of such a system to all viruses renders it unwieldy. Also, as a number bears no relationship to any property of the virus to which it is given, the difficulties in remembering what are the specific properties of viruses with such names as tobacco virus 18 or *Solanum* virus 17 are considerable. BENNETT (1939) has pointed out the many difficulties of a nomenclature based on numbers and suggested that names might replace numbers with considerable advantages. For example, *Nicotiana virus altathermus*, obviously tells more than *Nicotiana virus* 1. Such a name could readily be turned into a true Latinized binomial, if it is ultimately decided that viruses should be classified into genera and species. As an illustration, BENNETT suggests that tobacco mosaic virus might then become *Paracrystalis altathermus*, a name that really carries some information about the virus.

On the other hand, should it be decided that a nomenclature more in keeping with those in use by chemists rather than those in use by biologists should be applied to viruses, then the specific name could be given an appropriate suffix. The designation of a particular class of substances by a common suffix, for example the use of "ase" to denote enzymes, is an established practice in chemistry, and BENNETT suggested that the suffix "vir" might be adopted to denote virus, so that tobacco mosaic virus could become "altathermovir".

FAWCETT (1940) adopted the suffix principle and prepared a scheme that he claimed combined the best features of JOHNSON's, SMITH's and HOLMES' schemes. The rules are that the stem "vir" for virus is added to the Latin generic name of the genus in which the virus was first discovered and the specific name is taken from the list devised by HOLMES. As examples FAWCETT gives *Rubivir orientale* for raspberry streak virus and *Solanivir vastans* for potato yellow dwarf virus, but it is not clear what tobacco mosaic virus would become; presumably it would be *Nicotianivir Tabaci*, which, to say the least, would seem to emphasize the host plant unnecessarily.

VALLEAU (1940) prepared a key for the identification of 8 viruses commonly found attacking tobacco crops, and used the HOLMES type of name, except that he omitted the trinomial for strains. Also some of HOLMES' names were modified because VALLEAU rightly considered that HOLMES' "generic" names indicated false relationships; in this scheme tobacco mosaic virus, for example, becomes *Musivum Tabaci* and cucumber mosaic virus *Murialba cucumeris*.

Each of these schemes has some adherents, and some form of Latinized binomial system seems to be receiving increasing favour, but none has been unanimously accepted or put forward with any official support, or seems to have any overwhelming advantages over any of the others.

Because of the large number of synonyms for many viruses, an official catalogue of approved names would probably be very useful to virus workers and general plant pathologists. But until such a list has been prepared, an attempt to use any of the newly suggested systems necessitates listing synonyms after each name to ensure that the reader has at least some idea what virus is being referred to. To avoid the difficulty, in this book, as in the first edition, these systems will not be used, and the viruses will be described either under the names they were first given or under those used by workers who have studied them most.

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SYMPTOMATOLOGY

Viruses, being invisible, can be recognised only because of the visible effects they produce. Of these, the most characteristic and obvious is the ability to produce changes in the appearance of plants. Even this, however, is not a universal property. There are some plants which can be fully infected with a virus (or even with more than one virus) without looking at all abnormal. But the presence of a virus in such a tolerant plant, or carrier, can be demonstrated by infecting intolerant species, which will then show symptoms. It is possible that there are viruses to which all plants are tolerant, but for practical purposes this possibility can be ignored, because with present methods they would behave like, and be indistinguishable from, normal plant constituents. It will be realised, therefore, that symptomatology, although of great value in diagnosis in all branches of pathology, is especially important in virus work, for it is obvious that the recognition of the very existence of a virus depends on its ability to cause disease.

In describing symptoms, as with any other property of viruses, it is impossible to make any general statements to which there are no exceptions. Nevertheless, the virus diseases found at all frequently in nature have certain features in common which separate them sharply from most other infectious diseases. These result from the ability of most viruses to invade the whole of the vegetative parts of infected plants, producing what is known as a systemic infection. Consequently, symptoms tend to be scattered more uniformly over the whole of infected plants than they are in the more localised diseases caused by fungi or bacteria. The symptoms of virus diseases are often more closely simulated by the action of toxic substances or by root damage than by those caused by other pathogens. For example, the symptoms of potato leaf roll can be produced by fumigation with tetrachlorethane or by injuring the bases of potato plants; and small amounts of copper (CALDWELL 1935) and sodium chlorate (OWEN 1937) in the soil may produce typical mosaic symptoms in some plants. With virus diseases the symptoms are usually more definite in the young, actively growing portions than they are in tissues reaching maturity at the time of infection. Their infectivity clearly distinguishes them from diseases caused by toxins; also, unless the toxin is present continually, affected plants usually recover, but this rarely happens with virus diseases.

External symptoms:—Although plants react in a variety of ways to the presence of viruses, there are a number of symptoms produced with great frequency. Perhaps the commonest of these is an alteration in the colouring of the leaves. Instead of being uniformly green,

they have patches of light green, yellow or even white, conditions usually described as mosaics, mottles or yellows. Diseased plants are smaller than normal and the leaves are often greatly deformed. The deformation may take the form of an alteration in the outline, a crinkling or puckering of the surface, or a reduction of the lamina so that the leaf may consist of little more than the main vein and look like a tendril. Sometimes there is local hyperplasia. On the leaves the most common form is the production of ear-shaped outgrowths, the so-called "enations", while the lateral buds may proliferate excessively giving rise to a witch's broom effect. Necrosis, death of the tissues, which may be local or general, is extremely common in experimentally produced diseases, but is less so in those occurring naturally.

The production of any one type of symptom can rarely be taken as conclusive evidence for the presence of any specific virus. The same virus can often cause many kinds of symptoms, depending upon the species or variety of the infected plant and on the environmental conditions. Also, different viruses may cause almost identical symptoms in the same host.

Many diseases pass through three distinct phases. Firstly, there are visible local lesions formed at the points of entry of the virus into the plant. The virus then spreads through the plant and the next symptoms appear on the young leaves. These systemic symptoms are often of two kinds, those first formed differing widely from those produced later. Although this is perhaps the commonest sequence, it is by no means universal. Indeed, one virus may produce a disease showing all three phases in one susceptible species and a disease with only one or two phases in another. Some viruses even produce quite distinct diseases in different varieties of the same species. This variation in the symptoms produced by individual viruses can probably best be indicated by a few examples.

Most of the degeneration in the potatoes grown in the south and east of England is caused by potato virus "Y" (SMITH 1931). This is often difficult to diagnose as the virus causes different symptoms in different varieties. In some, for example Majestic and President, it causes leaf-drop streak (acropetal necrosis) a disease with three well defined phases. A few days after infection, the inoculated leaves develop local lesions consisting of black necrotic spots. About a month after infection, systemic symptoms appear. These consist of a blotchy mottle spreading from the veins of the uppermost leaves, which are also wrinkled and waved and generally have a crinkled appearance. At the same time black necroses appear on the undersides of the veins of leaves occupying an intermediate position on the stem. The necroses spread along the veins and pass down the petioles to the main stem, when the leaves collapse and shrivel. The falling of the leaves advances acropetally until all except those at the very top are affected. The next, and final, phase of the disease is shown by plants raised from infected tubers. The long, black veinal necroses and leaf-dropping are much less evident. Instead, affected plants are extremely dwarfed and brittle, the mottled leaves being twisted, waved and closely bunched together. As a contrast to this sequence of symptoms, in

other potato varieties such as Arran Victory, potato virus "Y" produces a disease with only one type of symptom. The inoculated leaves show nothing, and the systemic symptoms, in both the first and subsequent years of infection, consist merely of a mild crinkle. The leaves are slightly mottled and waved, but there are few or no necroses and no leaf-drop.

In tobacco, var. White Burley, potato virus "Y" produces yet another kind of disease. Usually no visible local lesions are formed, but the systemic symptoms pass through two distinct phases. About a week after infection, the areas around the veins of the youngest leaves become chlorotic. A few days later the vein-clearing fades, and the subsequent symptoms take the form of an interveinal mottling with pronounced dark green bands along the veins. In *Lycium barbarum* (DENNIS 1938), on the other hand, potato virus "Y" merely produces necrotic local lesions and gives no systemic infection.

Similar variations are found in the different diseases produced by potato virus "X". Many of the commercial English varieties, e.g., Majestic and Up-to-Date, and all American ones, carry this virus. Indeed, it is unusual to find individuals of these varieties, however healthy in appearance, that are not infected with at least one strain. In varieties such as Epicure, Arran Crest and King Edward, this virus causes a lethal disease, known as acro- or top-necrosis. The inoculated leaves develop black, necrotic spots, and within a fortnight the upper leaves become smothered with small necroses. The plants then rapidly die from the top downwards, and may be killed within a month. Many of the tubers set by such plants exhibit deep fissures or ulcers. The eyes are often necrotic and rarely sprout; those that do, give rise to minute, acutely necrotic plants that soon die.

In tobacco, potato virus "X" produces quite different effects. The inoculated leaves show local lesions consisting of a central, white necrotic spot surrounded by a number of concentric rings. Systemic symptoms, also of the ringspot type, develop in a few days, but after some time these decrease in intensity, so that any leaves produced a few weeks after infection may show only slight symptoms. A similar reduction in the severity of the disease with increasing length of time that plants have been infected is often seen with other viruses. The effect is probably most definite in species of *Nicotiana* infected with tobacco ringspot virus (PRICE 1932). Local, necrotic lesions develop in tobacco in about three days and severe necrotic rings appear on the young leaves after ten days. But after these early symptoms, any new leaves formed by the plants may look quite normal, or show only a slight mottling. Cucumber plants react somewhat similarly to this virus; soon after infection they show a fairly severe mottling with necroses, but new leaves formed after infection show none of these symptoms. Instead, numbers of enations are formed on the under surfaces, the upper surfaces often looking quite normal.

Pronounced symptoms are most commonly seen on the leaves and stems, but flowers and fruits also frequently show abnormalities,

especially when infected with viruses causing leaf mottling. The most well known flower change resulting from virus infection is probably breaking in tulips. Tulips raised from seed give self-coloured flowers, but after becoming infected with the virus the petals become variegated. The condition once established is permanent and bulbs taken from broken plants continue to give rise to mottled flowers. Broken tulips were often sold as new varieties in the past, e.g., Rembrandt is merely an infected Princess Elizabeth, and were much sought after. With the discovery that the condition was a result of virus infection, however, and could be produced at will, the demand for such plants has become less. Although broken tulips are a little less robust than normal ones and the leaves sometimes show faint mosaic symptoms, they do not seem to be at all seriously affected, and some have been in cultivation for more than three hundred years. In addition to tulips, variegation in flowers has also been described in *Nicotiana* species, sweetpeas, stocks, wallflowers and *Zinnia* when infected with various viruses causing mosaics.

A yellowish-green mottling of the fruit, accompanied by the production of wart-like projections, is produced by infecting cucumbers with cucumber virus 1, or with tobacco ringspot virus. Tomato fruits are affected by a number of viruses. Chlorotic rings and mottles are produced by viruses of the tobacco mosaic type and tomato spotted wilt virus, and irregular blotches or cracks by tomato streak virus.

It is unusual for roots of diseased plants to show any macroscopic changes, although they become infected. Indeed, in some diseases it seems that only the roots contain virus. Even then the roots show no external symptoms, although the aerial portions may. Examples of this are phony peach (HUTCHINS 1933) and tobacco necrosis (SMITH and BALD 1935). Affected peach trees are dwarfed, produce a reduced crop of small fruit, and have unusually rich green leaves and smooth bark, but the presence of the virus has not been demonstrated in any part of the plants except the roots. Tobacco plants which look quite healthy may contain a virus in their roots capable of causing a severe disease (tobacco necrosis) when introduced into the leaves. SMITH (1937) has shown that the leaves of normal-looking tobacco plants often develop severe necrotic spots when rubbed with extracts of their own roots. The spots may increase in size until the whole leaf is affected and dies, but it is extremely rare for the virus to move into uninoculated leaves. The two lowest leaves of a plant sometimes become naturally infected, but after these have shrivelled the plants again look normal. The virus can be recovered from the roots of plants that have been infected either artificially or naturally long after all symptoms in the foliage have disappeared. Black root of snap bean is exceptional, for in this disease external black streaks can be seen, which are correlated with histological changes in the vascular system (JENKINS 1940, 1941).

Localisation of symptoms is not uncommon in virus diseases produced artificially but is rare in those occurring naturally. Tobacco necrosis virus is peculiar in that it has a wide host range, but in no plants is it known to become systemic in the leaves. Other viruses

produce necrotic local lesions and no systemic infection in a few plants, the most well known example being tobacco mosaic virus in *Nicotiana glutinosa*, but these all have alternative hosts in which they become systemic. The symptoms of diseases restricted to local lesions are always of the necrotic type. They may be compared with the effects of *Puccinia graminis* in wheat varieties which are immune because of their hypersensitivity, the plant tissues being so susceptible that they die in advance of the spread of the pathogen. Both the rust fungus and the virus are obligate parasites and if isolated in areas of dead tissue will be unable to multiply.

In the brief description of the main types of symptoms of virus diseases that has been given above, one unwarranted generalisation has been made. It has been said that potato virus "X" or tobacco mosaic virus produce such and such symptoms in infected plants. This needs considerable qualification. The most that can be said is that a specific strain of a virus produces a certain type of symptom, for different strains of the same virus often produce quite dissimilar symptoms in the same host plant.

For example, the symptoms that have been given for potato virus "X" in tobacco are those caused by the S strain. Other strains do not cause ringspot, for strain G produces a barely distinguishable mottle while strain L produces a bright yellow mottle (SALAMAN 1933). Again, the potato varieties named as carriers of this virus may carry two or three strains but be severely affected by others.

Strains of tobacco mosaic virus may also produce widely different symptoms. The common strain in tobacco and tomato produces a faint green mottle, with only slight deformation of the leaves. The strain causing aucuba mosaic in these two hosts produces an extremely bright yellow mottle, whereas the strain causing enation mosaic of the tomato causes a condition indistinguishable from common tobacco mosaic in tobacco but acute deformity and the formation of many enations in tomato. All strains produce similar necrotic local lesions with no systemic infection in *N. glutinosa*.

The symptoms produced by different strains of one virus may differ much more than those produced by two quite unrelated viruses. For example, the symptoms in tobacco resulting from infection with aucuba mosaic virus much more closely resemble those produced by yellow cucumber mosaic virus than those of common tobacco mosaic.

Effect of environment on symptoms:— Even when the variety of the host and the strain of a virus are both specified it is impossible to make dogmatic statements about the symptoms unless the environmental conditions are also given, for changes in these greatly modify the appearances of infected plants. They may even determine whether a plant shows symptoms or behaves as a carrier, and whether infection is localised or general.

The two most important conditions affecting symptoms are light and temperature. In general, high temperatures tend to reduce the severity of the diseases, but the optimum temperature for symptom

production varies with different diseases. Crinkle and mosaic of the potato are suppressed completely by short periods of exposure to temperatures of over 20°C , whereas temperatures in excess of 35°C are necessary to mask the symptoms of tobacco mosaic. Reducing the temperatures to the more normal range of 15°C to 20°C usually results in the production of symptoms again, but sometimes long exposure of plants with tobacco mosaic results in a permanent alteration in the symptoms. The treated plants continue to show reduced symptoms, and others infected from them also show little signs of disease, although grown at normal temperatures. The growing of tobacco plants with mosaic at abnormally low temperatures, below about 7°C , also results in the masking of the symptoms (GRAINGER 1936) and the temperature range over which typical symptoms are obtained is from 10°C to 30°C . Over this range, increase of temperature or light intensity reduces the interval between inoculation and the appearance of symptoms. This is especially obvious in *N. glutinosa* infected with tobacco mosaic virus, the time interval between inoculation and the time of maximum rate of appearance of local lesions being 50% longer at 15°C than at 20°C . Increasing the temperature also increases the total number of lesions produced, it being about 30% greater at 20°C than that at 15°C (BEST 1936). The type of reaction of *N. glutinosa* to infection can also be changed by growing plants at different temperatures. At low temperatures the necrotic local lesions are small and remain quite discrete. But above 28°C they tend to increase in size and to run together, while at 35°C or higher there are no necrotic spots, the inoculated leaves showing merely faint chlorotic blotches, and full systemic infection is obtained (SAMUEL 1931). The development of definite symptoms in strawberry plants with yellow-edge depends on both temperature and soil moisture. Only when the air temperature is above 16°C and there is adequate moisture are symptoms easily detected, so that infected plants in the field can usually be identified with certainty only in the late spring or early autumn (KING and HARRIS 1942). Potato yellow dwarf is a striking exception to the generalisation that high temperatures decrease the severity of symptoms (WALKER and LARSON 1939). Below 16°C symptoms may be completely masked, but increasing the temperature above this increases both the speed at which symptoms appear and their severity. High soil temperatures may also prevent the sprouting of infected tubers that will grow normally at low temperatures. ✓

Growing virus-infected peach trees for some time at about 35°C has a rather different effect, for it seems to destroy the viruses inside the plants. Affected leaves retain their characteristic diseased appearance, but any new growth formed after this treatment is quite normal. KUNKEL (1936) has shown that peach trees suffering from yellows, rosette, little peach and red suture, all produce new healthy shoots if grown for some time at high temperatures. The length of time necessary varies with the temperature and the size of the tree to be treated, and is considerably greater for destroying the yellows virus in the roots of potted plants than for curing the tops only. KUNKEL suggests that the geographical distribution of peach yellows in the

U.S.A. may be determined by the temperature, for the disease is not found in the Southern States where temperatures of 35° C prevail for long periods, except in the mountainous regions of Virginia where summer temperatures are relatively low. The distribution of virus in peach trees with yellows, after the virus has been destroyed in the tops by heating but not in the roots, strikingly resembles that described by HUTCHINS (1933) for phony peach in which the virus is restricted to the roots. HUTCHINS' work was done in Georgia where the summer temperatures are high, and KUNKEL makes the interesting suggestion that the peculiar distribution of the virus in trees suffering from phony is not necessarily a result of the inability of the virus to infect the aerial portions, but that the virus in the tops is destroyed by the heat and is able to persist only in the cooler roots.

Reducing the light intensity has been found to reduce the severity of the symptoms of a number of diseases. Variegated *Abutilon* plants produce quite normal green leaves when grown in the shade. Tomato plants infected with sugar beet curly top virus under field conditions usually die, but SHAPOVALOV and LESLEY (1931) found that if plants were shaded they were more tolerant to infection and were then able to produce a crop. Aucuba mosaic virus in the summer causes a bright yellow mottle in tomato with only little deformation of the leaves but in winter the mottle is usually slight and the leaves considerably deformed, the laminae being greatly reduced giving a "fern-leaf" effect. Cucumber virus 1 also produces this effect in tomato plants, but with this virus the fern-leaf cannot be prevented by increasing the intensity of the illumination (AINSWORTH 1935)✓

BEST (1936) has found sunlight to have an inhibiting action on the rate of appearance of the local lesions produced by tomato spotted wilt virus in *N. Tabacum*. Continuous light also reduced the total number of lesions obtained, but no such effect was obtained with tobacco mosaic virus in *N. glutinosa*. BEST suggests that this may be the result of some photochemical action that inactivates the much more unstable tomato spotted wilt virus before it has developed sufficiently to cause visible lesions.

The condition of the host plant at the time of infection also can greatly affect the symptom picture. In general, the more vigorous and actively growing the plant is at the time of infection the more pronounced are the symptoms shown. Mature leaves rarely show definite symptoms unless they are actually inoculated. They then develop local lesions, systemic effects usually being restricted to leaves which are either still growing or are produced after infection. Many plants become increasingly resistant to systemic infection with age. Young tobacco plants develop a severe general ringspot when inoculated with a virulent strain of potato virus "X", but older, slowly growing plants usually produce many necrotic local lesions without any systemic symptoms. Similarly, tomato seedlings are quickly killed by tomato bushy stunt virus; slightly older plants show milder systemic symptoms consisting of occasional yellow blotches or necroses, and still older plants restrict infection to the inoculated leaves. Any condition simulating old age in a plant may lead to an increased

resistance to systemic infection; for example, an older tobacco plant that is still actively growing will become fully infected with potato virus "X" while a younger one that has become potbound and ceased growing will give only local lesions.

The presence of one virus in a plant may also have a great effect on the symptoms produced by a second. If the two viruses are related strains, infection with the second usually produces no further results. For example, tomato plants fully infected with tobacco mosaic virus and showing a faint mottle fail to develop the bright yellow mottle when inoculated with aucuba mosaic. On the other hand, if the two viruses are unrelated they may cause quite a different condition when present in a plant together from that when either is present alone. The potato variety President shows only a mottle when infected with potato virus "X" and normally carries virus "A", but when a plant carrying virus "A" becomes infected with virus "X" it develops a severe crinkle (MURPHY and MCKAY 1932).



FIG. 1. — The effect of dilution on the number of local lesions caused by tobacco mosaic virus in leaves of *Nicotiana glutinosa*. (Undiluted sap, 1:3.1; 1:10; 1:100; 1:1,000) (HOLMES, F. O., 1929, Bot. Gaz. 87, 39).

The use of symptoms in quantitative work: — Many of the properties of viruses, for example, their resistance to physical and chemical treatments, can be investigated only indirectly by determining the effect of such treatments on the infectivity of expressed sap. Advances in our knowledge of the properties of viruses, therefore, are greatly dependent upon the accuracy of the methods used to estimate the concentration of active virus in a given preparation. The production of systemic symptoms is of little value for this purpose, because it merely shows that there is sufficient virus in the inoculum to produce infection. Except for a slightly longer delay between the times of inoculation and appearance of symptoms, there is no significant difference in the systemic symptoms of a plant receiving a minimal infecting dose and one receiving many times this amount.

In early work the infectivity of different virus preparations was compared simply by diluting greatly and then inoculating the diluted samples to large numbers of plants. From the number of systemic infections obtained the relative concentrations of virus in the different preparations was estimated. By this method, large differences in virus contents were indicated but small differences could be detected only by using enormous numbers of plants.

So far no method has been devised to give a measure of the actual

number of infective units present in a virus preparation, but by using local lesions instead of systemic symptoms as indicators of infection it has become possible to compare the virus contents of a number of different preparations with considerable accuracy. This possibility was first indicated by HOLMES (1929), who found that certain species of *Nicotiana*, especially *N. glutinosa*, produced countable necrotic lesions when their leaves were rubbed with sap from plants suffering from tobacco mosaic. He showed that the number of lesions could be used as an indication of the amount of virus in the inoculum, for leaves inoculated with undiluted sap produced some hundreds of lesions per leaf whereas those inoculated with sap diluted 1 in 1,000 with water produced only an average of 10 or so. (Fig. 1).

HOLMES compared the method with KOCH's plate method for quantitative work with bacteria, and various workers have since drawn an analogy between the production of bacterial colonies on agar plates and of local lesions on susceptible leaves rubbed with virus preparations. In theory the two methods are very similar, but in practice they have important differences. Unlimited numbers of agar plates can easily be prepared that differ in no essential manner from one another, but it is impossible to obtain two plants that are identical in all respects. The production of a local lesion does not depend solely upon the presence of an active virus unit, but is the result of a reaction between virus and host that may be affected by a number of conditions. Also, the number of lesions produced, even at high dilutions, cannot be taken as the number of infective units present, in the same way as can bacterial colonies as the number of bacteria, for the virus is able to produce lesions only at suitable entry points and not equally over the whole surface of rubbed leaves.

HOLMES found that the numbers of lesions formed by different plants inoculated with the same sample of infective sap varied considerably. He also noted that there was a gradient of susceptibility in the individual leaves of each plant and that the method of inoculation affected the number of lesions, rubbing with cheesecloth dipped in sap being more effective than pricking with needles. These observations have been repeatedly confirmed, and later work has been largely devoted to refining HOLMES' original method in attempts to eliminate variations resulting from differences in host plants. SAMUEL and BALD (1933) showed that there was little variation in susceptibility between the opposite halves of the same leaves and that the accuracy of the method was greatly increased by making use of this fact. Since then most workers have compared the activities of two virus samples by applying them to opposite halves of the same leaves. But when more than two samples are being compared this method must obviously be modified. The simplest modification is to select one preparation as a standard and apply it to one-half of every leaf while the other half leaves are apportioned between the remaining solutions. Each preparation can then be compared directly with the standard and indirectly with any other on the basis of their individual differences from the standard.

YODEN and BEALE (1934) using statistical methods have examined the variation in susceptibility of *N. glutinosa* to tobacco mosaic virus in some detail. They found that the two halves of the same leaf reacted equally, but that there were significant differences between plants and between the individual leaves of one plant. To overcome these variations they suggest several experimental designs based on those used in agricultural field trials. The most useful of these is that of the Latin square, in which the inoculations are arranged so that each preparation appears once on each plant and once at the same leaf position. An example in which six treatments are to be compared is given below. The plants are first decapitated and then stripped of all but six leaves occupying the same relative positions on the stem. The decapitation not only makes the plants easier to handle but also reduces the variation in susceptibility. The thirty-six leaves are now labelled so that each treatment occurs once on each plant and once at each leaf position. In the example given the columns represent individual plants and the rows leaf positions.

A	F	B	E	D	C
D	C	F	A	B	E
E	A	D	B	C	F
C	E	A	D	F	B
B	D	C	F	E	A
F	B	E	C	A	D

This design has the further advantage that the square can be "split" for half-leaf comparisons. For example, supposing two virus preparations are being compared over a range of six different dilutions, then each letter will represent a dilution, the preparations at each dilution being applied to the opposite halves of the same leaves. In this way not only is the effect of dilution determined but direct comparisons of the two preparations at every dilution are also obtained with the minimum of experimental error.

The Latin square design is the most convenient when working with plants such as *N. glutinosa* which produce a large number of inoculable leaves, but it obviously cannot be used when the number of treatments exceeds the number of available leaves per plant. Large numbers of treatments can be compared by modifications of the half-leaf method. One of these, comparing all treatments with the same standard, has already been indicated. Another is to pair all the treatments in every possible way and then apply the pairs to the opposite halves of the same leaves. YODEN (1937) has pointed out the value of the incomplete block method of experimental design for comparing the infectivities of a large number of treatments. Each plant represents an incomplete block and the treatments are apportioned among the plants in such a way that every treatment is compared with every other in the different blocks. Two possible arrangements for seven treatments on plants with only three available leaves are

A	B	C	D	E	F	G	A	B	C	D	E	F	G
B	D	F	E	G	A	C	B	C	D	E	F	G	A
C	F	E	A	B	G	D	D	E	F	G	A	B	C

The rows resemble those in the Latin square and are complete blocks with all treatments arranged to be applied once to each leaf position, whereas the columns are incomplete blocks for all the treatments are not represented in each plant.

Even if comparisons between the infectivity of virus preparations are made solely by direct comparisons between the total lesions produced by each preparation, experimental designs of these types greatly increase the accuracy of the local lesion method. Furthermore, if the results are treated statistically, tests for significance can be applied and the real value of any differences assessed. And, with the incomplete block design, variation in lesion-numbers resulting from differences in the susceptibility of the host plants can be determined and eliminated.

The actual numbers of lesions produced by different virus samples may give little indication as to their relative virus contents; for the number of lesions is directly proportional to concentration over only a limited range. For practical purposes the dilution curve obtained by plotting the number of lesions produced against dilution of the inoculum can be considered as consisting of three portions. At high concentrations there is a flat portion where dilution has little effect on the number of lesions. At intermediate concentrations there is an approximately straight line portion where dilution is accompanied by an equivalent reduction in the number of lesions, while at low concentrations the reduction in lesion numbers is again less than the change in dilution.

All viruses give similar dilution curves, for the numbers of lesions produced by concentrated inocula always approach a limiting value. For accurate comparisons of the relative virus content of different samples, therefore, it is obviously necessary that tests should be made over the portion of the curve represented by the straight line. To ensure this and to avoid testing at concentrations where relatively large differences in virus content produce only small changes in the number of lesions, it is usually best to test unknown samples over a fairly wide range of dilutions.

The upper limit set to the number of lesions produced by a leaf has most often been attributed to the number of entry points available, but it seems probable that other factors are also involved. It is clear that more lesions than the number of entry points cannot be obtained, and the dependence of the limiting value on the method of inoculation and on the condition of the plants at the time of inoculation can be explained by their effect on the number of entry points. But BEST (1936) has shown that the limiting number can be affected by the conditions under which the plants are placed after infection, and this can hardly alter the number of entry points.

The conditions for satisfactory quantitative work with the local lesion method can therefore be summarised as follows. Plants as uniform as possible should be used and should be treated identically both before and after infection. An experimental design that reduces errors coming from variations in susceptibility should be used and the results analysed statistically to determine their significance. The samples of virus being compared should be alike in all respects except

virus content and should be tested over the concentration range in which the infectivity is most nearly proportional to the dilution. A standard method of applying the samples to the leaves should be used.

SAMUEL (1930) has shown that the method of inoculation has a definite effect on the number of lesions produced. In general, light rubbing, producing no obvious sign of damage to the tissues, is most effective. The methods most used consist of rubbing the whole leaf with either the forefinger or with a piece of muslin dipped in the fluid to be tested. SAMUEL suggested using ground-glass spatulae with the blade made sufficiently long to cover half the width of the leaves to be inoculated. When dipped in the experimental fluids these would pick up the same amount each time and by drawing them once from end to end of the leaf the amount of rubbing would also be controlled. In the writer's opinion the forefinger gives the best results for by this method it is easiest to control the degree of rubbing, but the necessity for repeated and thorough washing of the hands between each test is a great disadvantage. A much more even pressure is obtained if the leaf to be inoculated is supported on a piece of soft rubber covered with clean paper.

Immediately after inoculation the leaves should be washed with a stream of water. This treatment does not prevent infection. On the contrary, it often increases the number of lesions produced, by removing any toxic substances in the inoculum which might affect the leaf tissues.

Although the local lesion method is used generally for measuring the relative virus contents of different samples, it must be stressed that what it actually measures is not this but the relative infectivities of the samples. It is of some importance that the difference between the two should be clearly appreciated. If the samples under test are equivalent in all respects other than virus content and are tested at suitable dilutions, then it is to be expected that the difference in their infectivities will be an indication of their difference in virus content. But if the samples have been treated differently or contain different substances, or different amounts of the same substances, then it is by no means certain that the numbers of local lesions they cause will at all accurately reflect their relative virus contents. A reduction in infectivity may indicate a reduction in virus content. On the other hand, it may result from the presence in the inoculum of something which decreases the susceptibility of the host plant, or which unites with the virus to form a non-infective complex without necessarily destroying the virus. Or it may be produced by a treatment that has increased the size of the infecting units. An aggregate of ten infecting units will be no more infective than a single infecting unit. Although the aggregate contains ten times the weight of virus, it will produce a single local lesion. Two virus preparations with an equal concentration of virus may give widely different numbers of local lesions if one has been subjected to any treatment causing the virus particles to aggregate. Because of the effects of such factors on infectivity, it is essential in using the local lesion method as a measure of the relative virus contents of different preparations that

the preparations should be equivalent in all respects other than virus content.

It is known that some substances, for example, proteins, salts, acid, and some enzymes, which do not destroy tobacco mosaic virus, if added to the inoculum greatly affect the number of lesions formed. The effects of these will be discussed in more detail later in the book, and they are mentioned here merely to stress the fact that samples being tested for their relative virus contents should be as similar as possible.

A number of workers have attempted to devise methods whereby it would be possible to distinguish between substances reducing infectivity by acting directly upon the virus and those reducing the susceptibility of the host plant. These are based on the different effects such substances would be expected to have upon the form of the dilution curve. STANLEY (1934) and CHESTER (1934) both suggest that substances affecting the plants, when added in constant amounts to various dilutions of virus, will cause a fairly constant decrease in the number of lesions at all dilutions, whereas those affecting the virus will produce a greater decrease when added to dilute preparations than when added to concentrated ones. CALDWELL (1936) agrees with this, except that he suggests that substances affecting the plant will have less effect at high than at low dilutions. Distinctions between the two types of action should therefore be demonstrated by doing infection tests at various concentrations of virus with a constant amount of the substance added to each virus sample. If the reduction in number of lesions increases with decreasing virus concentration, the substance is acting on the virus; on the other hand, if the reduction remains constant or decreases, it is acting on the plant. Unfortunately, in practice these methods rarely give clear-cut results, and there is considerable disagreement between different workers who have used them as to whether some substances are having one or other action; more often than not, the only conclusion is that they are having both.

By analogy with the plate method of estimating bacteria, YODEN, BEALE and GUTHRIE (1935) have derived a formula which they consider represents the quantitative relationships of the concentration of virus to the number of lesions produced. It is

$$y = N(1 - e^{-ax})$$

where y is the number of lesions obtained with any given relative concentration x of virus. N is the maximum number of lesions obtainable and a is a constant, representing for the undiluted extract the average number of virus particles per entry point, while e is the natural base of logarithms. They claimed that this equation could be applied to all dilution curves. BALD (1935, 1937*a*) independently arrived at a similar formula but stated that only purified virus preparations gave dilution curves that could be fitted to the equation. By fitting data from dilution experiments to these formula it should be possible to decide whether a substance was affecting the host or the virus content by determining whether a change in N or x most nearly fitted the results. However, BALD (1937) later found that even

purified preparations often gave dilution curves that deviated from the suggested formula. It is now known that tobacco mosaic virus can aggregate and BALD suggested that the deviations were a result of such aggregates breaking up into smaller infective units when diluted.

The local lesion method is used exclusively by most workers in quantitative virus work, and without it progress would have been considerably slower. In practice, under adequately controlled conditions, it does provide a method whereby relative virus concentrations can be estimated with a reasonable degree of accuracy. The production of lesions is affected by so many factors other than virus content, however, that caution is needed in interpreting results, and it would be unreasonable to expect a simple relationship between lesion number and virus content, or even one that could be represented by a single equation. Alternative methods of quantitative working, not subject to such variations, but open to other objections, are described in the chapter on the serological reactions.

Starch lesions:— The local lesion method was discovered and largely evolved with tobacco mosaic virus and *N. glutinosa*, but it is now widely used with other virus-host combinations. In America *Phaseolus vulgaris* var. Early Golden Cluster is often used instead of *N. glutinosa* for lesion work with tobacco mosaic, as it gives equally good lesions and is raised much more easily and quickly.

Necrotic local lesions and no systemic infection are the ideal for quantitative work, as such lesions remain discrete and are easily counted. Some of the virus-host combinations giving such a result, which have been used extensively for this purpose, are tobacco necrosis virus and *Phaseolus vulgaris*, tomato bushy stunt virus and *N. glutinosa* and cucumber virus 1 and *Vigna sinensis*. Some viruses can be worked with by the local lesion method although they do give systemic infection in the hosts, for at the site of inoculation they produce visible lesions that remain discrete sufficiently long to be countable. Examples of these are the severe strains of potato virus "X" in tobacco and *N. glutinosa* and tobacco ringspot virus in tobacco.



FIG. 2a. — Types of local lesions produced at different times by severe etch virus on tobacco. — a, Starch-iodine lesions; this leaf showed nothing until decolourised and stained with iodine.

Many viruses produce either no visible local lesions or else merely chlorotic spots too vague to be counted. At first sight it would appear that these are not amenable to the local lesion method, but by varying the technique it can be used, though not so satisfactorily as

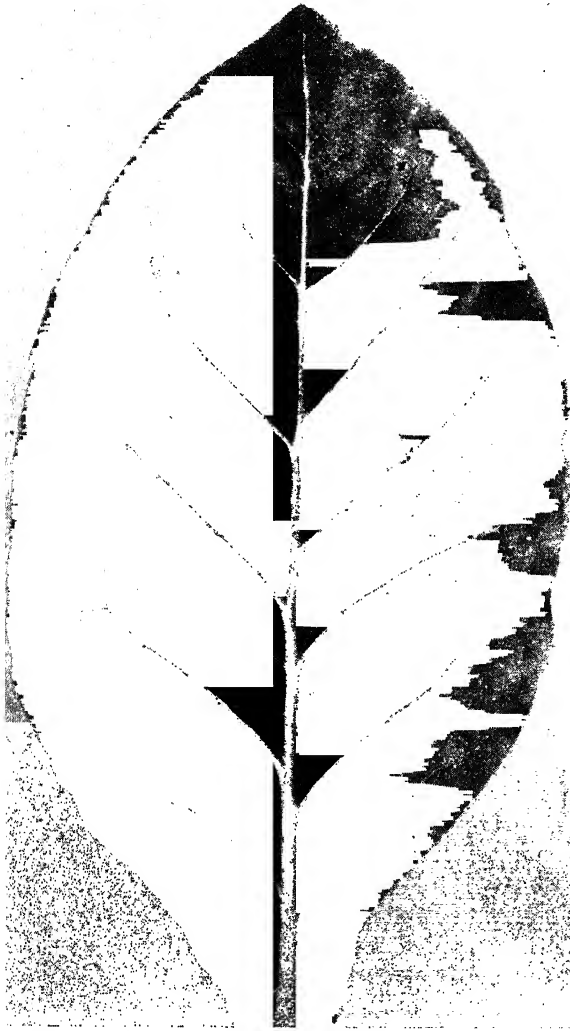


FIG. 2*b*. — Types of local lesions produced at different times by severe etch virus on tobacco. — *b*, Local lesions in the form of necrotic rings.

with those producing directly visible lesions. HOLMES (1931) showed that tobacco leaves inoculated with tobacco mosaic virus developed slight yellowish areas, which showed up much more definitely if the leaves were decolourised with alcohol and then stained with iodine. HOLMES used two methods. In one, the leaves were taken off the plant in the evening, decolourised and stained, when the general

leaf stained more deeply than the spots. In the other, the plants were placed in the dark for some hours before the leaves were taken off, when the lesions stained more definitely than the unaffected tissues.

HOLMES was able to correlate these effects with the presence of



FIG. 2c. — Types of local lesions produced at different times by severe etch virus on tobacco. — c, Local lesions in the form of diffuse chlorotic spots.

the virus for he found that the general leaf tissue was not infectious whereas the spots were. It seems therefore that the presence of this type of virus has two distinct effects. It reduces normal starch formation during light periods and also inhibits the movement of carbohydrates when plants are placed in the dark.

These effects have now been found to apply fairly generally to viruses causing mosaics, and by making use of them it is possible to obtain reasonably accurate lesion counts with viruses that have no very definite visible effects on the inoculated leaves. Usually the second method of leaving the plants in the dark before picking the leaves gives the better results, for the lesions are then much more clearly differentiated from the normal leaf tissue. The technique most suitable is to place the plants in a dark room overnight. In the morning the leaves are picked, killed by being plunged into boiling water and then decolourised with ethyl alcohol. They are then washed in water and stained in a solution of iodine in potassium iodide.

Under favourable conditions this method shows up the lesions quite clearly and they can be as easily counted as those produced by potato virus "X" in tobacco. It is, however, even more influenced by environmental conditions than the normal lesion method. The length of time for which the plants are left in the dark must be sufficient for the starch to leave the normal leaf tissue but insufficient for it to leave the lesions. This time will vary both with the temperature at which the plants are stored and with the amount of starch in the plants, which in its turn will vary with the environmental conditions of the day when the plants are placed in the dark. It is especially important that the test plants should all be equally illuminated, for shading of a leaf will prevent completely the production of starch-iodine lesions.

The length of time between infection and testing is also of great importance. If it is too short, the differences between the lesions and the normal leaf tissue will be insufficient for the lesions to be clearly differentiated. If it is too long, the virus will have spread considerably from the points of entry, the lesions will tend to coalesce and cease to be countable. The presence of the viruses in any part of the leaves and not only at the site of entry seems to affect the rate at which starch disappears when the plants are placed in the dark. The starch-iodine test can therefore be used to demonstrate the manner in which viruses move through the plant from entry points, as well as merely indicating the entry points themselves (Figs. 43 and 44).

The following viruses give reasonably good starch lesions in tobacco plants; tobacco mosaic, cucumber 1, *Hyoscyamus* 3, severe etch, potato "Y", and strains of potato "X" producing mottles. It is highly probable that numerous other viruses producing systemic mottles would also give them either in tobacco or some other host. Under standardised conditions of light and temperature this method might be made as accurate as the more usual method where lesions are visible without any staining. But under the fluctuating conditions of ordinary glasshouses the method is rather a hit-or-miss one, the results depending a good deal upon the judgement of the worker, and complete failures are not infrequent. Some of these viruses sometimes also give clearly countable local lesions, especially in the spring and autumn. Tobacco etch viruses, for example, usually produce no visible reaction on the inoculated leaves unless they are

treated with iodine, but occasionally they produce easily distinguished necrotic rings or distinct chlorotic spots (Fig. 2). These variations in local reaction depend on weather conditions, but the exact conditions determining them are unknown.

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Chapter III

SYMPTOMATOLOGY (*Contd.*)

Intracellular inclusions:—Internal changes of two kinds are frequently found in plants suffering from virus diseases. The first change consists of modifications of the normal tissues or cell contents, and the second in the production of peculiar intracellular inclusions not present at all in healthy plants. The second is the more characteristic, for similar bodies have not been found accompanying any infectious diseases except those caused by viruses. They have been seen in both animals and plants suffering from a number of different virus diseases, and their etiological significance has been a subject of continual speculation.

It is not doubted that they are a direct result of virus infection, but some workers have regarded them as stages in the life history of causative organisms and others merely as masses of coagulated plant protoplasm. Workers on animal viruses have in recent years adopted a different view. The use of ultra-violet light and microscopes of high resolving power has shown that the inclusions of some diseases are aggregates of numerous elementary bodies of approximately the same size as that estimated for the virus particles themselves by filtration experiments. The inclusions in infected animals have therefore been looked upon as visible aggregates of virus particles, although no adequate reasons have been advanced to explain the aggregation in the infected cells of particles forming quite stable suspensions *in vitro*.

Although the intracellular inclusions appear to be specific to virus infections, occurring in some with sufficient frequency to be of considerable diagnostic value, they have not been found accompanying all virus diseases. Their production depends more upon the infecting virus than upon the host plant infected; for example, they have been found in a large number of species infected with tobacco mosaic virus but in none of the same species infected with cucumber mosaic virus. The host plant, however, plays some part, the formation of inclusions being correlated with external symptoms. A virus may produce many inclusions in all species in which it causes mosaic symptoms, but it is unusual for it to produce them in hosts in which it is carried or causes severe necrosis. The presence of inclusion bodies in diseased plants is, therefore, strong evidence that the cause is a virus, but their absence cannot be taken as evidence to the contrary. More especially is this so, as inclusions often persist only for a limited period during which affected plants show pronounced symptoms.

Tobacco mosaic and related diseases:—Most work on the intracellular inclusions in infected plants has been done with strains of

tobacco mosaic virus, for they were first found and occur with great frequency in these plants. In 1903 IWANOWSKI described two kinds of inclusions in the cells of plants suffering from tobacco mosaic, one, amoeboid-like bodies, consisting of amorphous material, and the other flat, crystalline plates. These observations have been repeatedly confirmed, but interest has centred chiefly on the amorphous type of inclusion, for at first sight this differs more definitely from the constituents of normal cells. Crystalline material is common in both healthy and infected plants, and the plate-like inclusions, although recognised as peculiar to the infected tissues, have been regarded by all workers in the past as merely reaction products of the cell to the disease, with no etiological significance. GOLDSTEIN (1924, 1926) described both inclusions in great detail, and aptly summarised the conflicting views on the nature of the amorphous inclusions by naming them X-bodies.

The inclusions are most evident in the epidermal and hair cells and are conveniently examined in living preparations made by stripping the epidermis from the leaf. The X-bodies resemble compact masses of cytoplasm. Typically they are rounded or oval, but they may have a quite irregular outline, for as they are carried round the cell by the streaming cytoplasm they readily change their shape on coming in contact with the cell wall or nucleus. They vary in size considerably; in large cells the diameter may be as much as 30μ , in small ones as little as 5μ . They are partially translucent and granular, and usually contain one or more clearly visible vacuoles. GOLDSTEIN claims to have seen external membranes, but HENDERSON SMITH (1930) and SHEFFIELD (1934) were unable to confirm this.

The X-bodies formed by the different strains of tobacco mosaic virus differ slightly in appearance. For example, those formed by aucuba mosaic virus in *Solanum nodiflorum* are more definite entities than the tobacco mosaic bodies, and their granular character is much more pronounced. SHEFFIELD (1931) has studied in great detail the manner in which these bodies are formed. Soon after the cells become infected the rate of cytoplasmic streaming is increased and minute granules appear in the cytoplasm. These are carried passively around the cell, and when two particles meet, they fuse together to form larger bodies. The process of fusion continues until all the particles are contained in one, or occasionally two, granular masses. These masses then round off, become vacuolate, and appear less granular. They frequently contain mitochondria and oil globules, which are acquired during the circulation around the cell. The fully formed X-body and the nucleus are often found in contact, but the association is an accidental one (*see frontispiece*). It is a consequence of the tendency of circulating particles to accumulate at the junction of cytoplasmic strands, and several usually converge on the nucleus.

SHEFFIELD's results clearly showed that the X-body was neither a breakdown product of the nucleus nor an organism of the amoeboid type. The latter claim was often made previously by workers who claimed that the body possessed the power of autonomous movement,

grew in size, put out pseudopodium-like projections, or divided by fission. The bodies move about no more than any other of the cell



FIG. 3. — Cell from hair of *Solanum nodiflorum* infected with aucuba mosaic virus. *Below*, a needle has been inserted through the transverse septum into the cell, leaving the inclusion body unaffected. *Centre*, this photograph was taken 15 seconds after the body was punctured by the needle. The body is rapidly changing its consistency, forming a mass of clear bubbles in which a few granules are visible. *Above*, photographed 10 minutes later. The inclusion has almost disappeared, only a few scattered granules being visible. $\times 300$. (SHEFFIELD, F. M. L., 1939, Proc. Roy. Soc. B, 126, 529).

contents such as nuclei, and the other effects claimed can satisfactorily be explained by the mode of formation described. A small aggregate of particles joining up with a larger may present an appearance simulating pseudopodia. Also, two large masses may come together but

fail to fuse completely, and the two may later separate again. Both the fusion and the separation will give appearances superficially similar to multiplication by division.

Although SHEFFIELD was unable to see any signs of an external membrane to the bodies, she has obtained results suggesting that there may be one (1938). She has been able to remove the bodies intact from infected cells and subject them to various osmotic pressures. In salt solutions of 0.1 M the body persists indefinitely, at 0.07 M it disintegrates slowly, while below 0.05 M disintegration occurs rapidly. The bodies also disintegrate when mechanical pressure is applied to the cells containing them or when they are pricked with a micro-needle. With these treatments they break down into particles or granules, morphologically indistinguishable from those that



FIG. 4. — Hair cells of *Solanum nodiflorum* containing crystalline material produced from disintegrating amorphous inclusion bodies. — *a*. Photographed in ordinary transmitted light. — *b*. Photographed between crossed Nicol prisms. The crystals in the left-hand cell are lying edgewise and are birefringent. $\times 450$. (BAWDEN, F. C. and SHEFFIELD, F. M. L., 1939, *Ann. Appl. Biol.* 26, 102).

fuse together to form the body, which are soon dispersed throughout the cell (Fig. 3).

The fully formed X-body of aucuba mosaic persists in the cell for a relatively short time. About a fortnight to a month after formation, crystals appear within the body and gradually the whole mass loses its amorphous nature and becomes crystalline. The crystalline masses produced in this way do not seem to differ in any essential manner from the crystalline inclusions described below, which are formed without the intervention of the X-body (Fig. 4).

Although the X-bodies are so susceptible to changes in osmotic pressure, they are extremely resistant to pH changes. They are unaffected by the addition of acid to pH 2 and are preserved by the ordinary fixation methods. Microchemical tests have been made on them, and they give all the usual protein colour reactions. Apart from occasional oil globules enclosed, they do not give any positive

test for fats. They are sharply differentiated from the nucleus if stained by Feulgen's reagent, for the nuclei take on a deep red colour, while the bodies are unaffected (*see frontispiece*).

The X-bodies produced by other strains of tobacco mosaic virus look slightly different, but give identical colour reactions. They also break down when pricked with a micro-needle (LIVINGSTONE and DUGGAR 1934) and are unaffected by weak acids. There is no reason to think that they differ significantly from those of aucuba mosaic, but SHEFFIELD (1934) was unable to follow their method of formation and disappearance as she did with the latter.

The crystalline inclusions differ in many ways from the X-bodies and appear to be similar regardless of the infecting virus strain. They

may occur either alone or in the same cell as the X-bodies, and in infections with aucuba mosaic virus they may be produced either directly without the previous formation of X-bodies or indirectly from the degenerating X-bodies. They are best examined in fresh living cells, for fixation either destroys them or alters their appearance.



FIG. 5. — Hair cell of *Nicotiana glauca* infected with tobacco mosaic virus. Two platelike crystals are seen, one being almost a perfect hexagon. $\times 450$. (BAWDEN, F. C. and SHEFFIELD, F. M. L., 1939, Ann. Appl. Biol. 26, 102).

The crystalline plates are very variable in shape. Some are quite irregular, others are perfect hexagons (Fig. 5), but intermediate grades with one or two angles of 120° are the most common. They are colourless and transparent, and have a refractive index higher than the cell sap. They are undoubtedly true crystals with a three-dimensional regularity, for as they slowly turn over in the sap they show both side and end faces. When seen edgewise they are rectangular, often showing faint striations, especially if the untreated crystals are examined in polarised light. IWANOWSKI (1903) showed that the plates developed striations and seemed to be composed of needle-like crystals if acidified, a feature responsible for the name "striate-material" that has been applied to this type of inclusion.

When viewed between crossed Nicol prisms the appearance of the plates depends on whether they are lying flat or edgewise. If edgewise they are highly birefringent with straight extinction, but if flat they are not birefringent (*See Chapter 10*). This difference is shown in Fig. 6*a* and *b*. As many of them also have angles of 120° , it is highly probable that they are true hexagonal crystals, for these are birefringent only when viewed along the transverse axes. The presence of the birefringent cell wall, coupled with the small size of the crystals, makes a critical examination in polarised light difficult, and proof of the crystal class by means of an interference figure has not yet been obtained (BAWDEN and SHEFFIELD 1939).

The crystals also give all the usual colour reactions for proteins, but their instability makes them difficult to examine in detail. Any

damage to the cell containing them, the addition of acid or pricking with a micro-needle, causes them to break down into small needles or to dissolve (Fig. 7). Early in infection several crystals can often be found in a single cell. Each then may be definitely hexagonal, but later they coalesce to form a single, rather shapeless mass.

Intracellular inclusions have been found in the roots, stems, leaves and flowers of infected plants, but they are most common in the leaves, especially in the epidermal cells. They cannot be found at all times or necessarily in all infected cells. Some parts of the leaf may have inclusions in nearly every cell and a nearby part have none. In good growing conditions the first appearance of incipient X-body formation can be seen a week or so after infection, at the same time as external symptoms become evident in the young leaves. The inclusions rapidly increase in number, reaching a maximum about a month after infection. After a further month the X-bodies begin to disappear, those of aucuba mosaic being replaced by crystals. The crystalline inclusions persist for longer than the amorphous bodies, but after a few months of infection both types have usually disappeared.

The exact association of the inclusions with symptoms is not clear. Some workers state that they have found them only in chlorotic areas, and others that they occur also in the intervening green areas. In tobacco infected with a masked strain of tobacco mosaic virus, BEALE (1937) states that the crystalline inclusions are definitely restricted to the occasional scattered areas that show symptoms. Tobacco mosaic virus produces inclusions in all hosts in which it causes systemic infection and symptoms, but SHEFFIELD (1936) found none in the necrotic local lesions in infected *N. glutinosa*.

It seems that over periods of years the type and relative proportions of different kinds of inclusions produced by strains of tobacco mosaic virus can alter appreciably. In 1930 and 1931 HENDERSON SMITH and SHEFFIELD found that aucuba mosaic virus produced large numbers of X-bodies in *Solanum nodiflorum* and that the crystalline plates were formed only from disintegrating X-bodies. Crystal-like spikes, often as long as the cell, were also frequently seen. Between 1930 and 1940 no spikes were found and only few amorphous bodies;

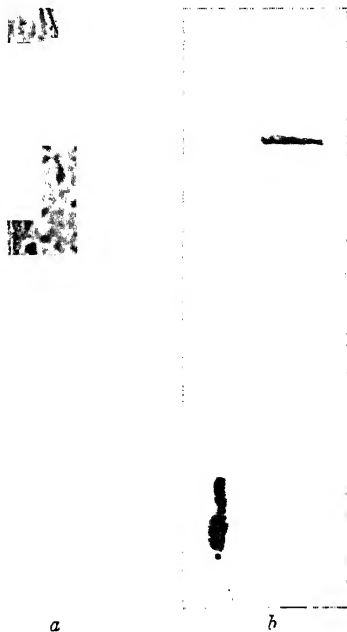


FIG. 6.—Hair of *Lycopersicon esculentum* infected with tomato enation mosaic virus. In the upper cell an irregularly shaped, crystalline inclusion is seen lying flat; in the lower cell a similar inclusion is seen lying edgewise. — *a*. Photographed by transmitted light. — *b*. Photographed between crossed Nicol prisms. The cross cell wall and the inclusion lying edgewise are birefringent, but the inclusion lying flat is not. $\times 450$. (BAW-DEN, F. C. and SHEFFIELD, F. M. L., 1939, *Ann. Appl. Biol.* 26, 102).

instead the infected plants contained large numbers of crystalline plates, produced as such and not indirectly from disintegrating X-bodies. In 1940 the spike-like body was found again with great frequency, as well as a number of other, previously undescribed, fibrous forms (KASSANIS and SHEFFIELD 1941). These consisted of spindle-shaped bodies, masses of short needle-like fibres, and long fibres often curved into figures-of-eight (Fig. 8). The reasons for these variations are unknown, but KASSANIS and SHEFFIELD suggest that weather conditions may be the controlling factor.



FIG. 7. — Epidermal cells from Turkish tobacco plant infected with tobacco mosaic virus after treatment with dilute HCl. The crystalline plates previously present have been replaced with numbers of paracrystalline needles. $\times 660$. (BEALE, H. P., 1937, Contrib. Boyce Thompson Institute 8, 413).

Possible relationship between tobacco mosaic viruses and the intracellular inclusions: — SHEFFIELD's results (1931, 1934) suggested that the X-bodies of tobacco and aucuba mosaic consisted of material coagulated and precipitated from the normal plant cytoplasm, and the view became generally adopted that both kinds of inclusion were plant reaction products. Recent work has necessitated a considerable modification of this view. Many of the properties of tobacco mosaic virus have been investigated in considerable detail and a knowledge of these makes it possible to put forward a possible explanation for the formation of both kinds of inclusion. In doing this it will be necessary to refer briefly to properties of the virus which are not described in detail until later chapters.

Neutral solutions of purified tobacco mosaic virus are faintly opalescent and show a slight satin-like sheen. On the addition of acid both the opalescence and the sheen increase. Between about pH 4 and pH 3 the virus is insoluble and precipitates in the form of microscopically visible needle-shaped bodies. Below pH 3 the preparations clear as the protein again dissolves, and below about pH 1 the virus is destroyed. When examined in polarised light the needles are found to be birefringent. BEALE (1937) has pointed out that the needles of the precipitated purified virus closely resemble in appearance the needles or "striations" produced by IWANOWSKI (1903) and GOLDSTEIN (1924) by adding acid to cells containing crystalline intracellular inclusions. She has also further shown that the pH stability and solubility ranges of the needles produced in the cells by the breakdown of the inclusions, are approximately the same as those of the purified virus *in vitro*. It seems highly probable, therefore, that the virus forms a major part of the crystalline inclusions.

However, some explanation is needed to explain why the virus should settle out in a solid or quasi-solid form in the cell sap. Purified tobacco mosaic virus is miscible with water in all proportions and does not settle out when solutions are concentrated. The fluids merely become increasingly viscous, ultimately turning to jellies when the solid content is raised to over 10%. A solid phase, in



FIG. 8. — A hair cell from a tomato plant infected with aucuba mosaic virus containing long, fibrous inclusion bodies curved to form figures of 8. $\times 450$. (KASSANIS, B. and SHEFFIELD, F. M. L., 1941, *Ann. Appl. Biol.* 28, 360).

the form of the needles, separates only if acid or if much salt ($1/5$ th. saturation with ammonium sulphate) is added. As the virus content of infective sap is only about 0.3%, the pH of the sap of uninjured cells is 6 or higher, and the salt content is far too small to precipitate the virus, it is highly improbable that the inclusions can be crystals of pure virus, for there is no apparent reason why virus should come out of solution in these conditions. It seems reasonable to assume, therefore, that the virus has united with some substance to form a complex insoluble at the pH and salt content of the cell sap.

Insoluble complexes of this kind can be formed by the purified virus (BAWDEN and PIRIE 1937*b*). When neutral solutions of certain protamines and histones, the one most studied being clupein, are added to neutral solutions of strains of tobacco mosaic viruses, material with a pronounced sheen immediately separates. The precipitated material consists of microscopically visible needles closely resembling the needles produced by acidifying either the inclusions or neutral solutions of the viruses (Fig. 9). The insoluble complexes contain less than 5% of clupein, which is apparently too little to affect the regular arrangement of the virus particles. Their solubility varies



FIG. 9. — Insoluble mesomorphic fibres produced by the addition of a neutral solution of clupein sulphate to a purified preparation of tomato aucuba mosaic virus. $\times 450$. (BAWDEN, F. C. and SHEFFIELD, F. M. L., 1939, *Ann. Appl. Biology* 26, 102).

greatly with variations in salt content and pH . They dissolve at all pH values at which the virus itself is soluble in salt solutions more concentrated than $M/10$, but the minimum amount of salt required for solution varies with the pH , and is greater at pH 5.5 than at pH 6. As protamines and histones are known to be constituents of most cells, it is possible that complexes of this type would occur in the infected plants. If the crystalline inclusions are complexes of the type suggested many of their properties can be explained. The sap of young, actively growing plants has a relatively high pH value and low salt content, conditions in which the complexes would be insoluble and tend to settle out. But the sap of older plants is more acid and has a greater salt content, conditions tending to render the complexes more soluble. The formation of the inclu-

sions in young plants early in infection and their disappearance with increasing length of infection could thus be explained by the changes in the composition of the cell sap. Their disappearance or changing into needles if the cell is injured or if acid is added could also be explained by the solubility relationships of such complexes. The effect of these two treatments is in all probability the same, for injury will reduce the pH value. With slight increase in the acidity and constant salt content, the solubility will increase and the inclusions will dissolve and disappear. With a greater increase in acidity, the acid precipitation point of the virus itself will be reached and the



FIG. 10. — *Nicotiana Tabacum* infected with tobacco ringspot virus. Epidermal cells from beneath a vein containing many small crystalline blocks. $\times 450$. (BAWDEN, F. C. and SHEFFIELD, F. M. L., 1939, *Ann. Appl. Biol.* 26, 102).

virus will separate out again as needles. Tobacco mosaic virus also forms fibrous precipitates with other substances, which more resemble normal cell contents than do protamines and histones. The addition of neutralised nicotine to a virus solution containing salt causes an opaque, fibrous precipitate to separate after a few hours. The precipitate disappears if the fluid is shaken, but it reforms when the preparation is again left undisturbed. Arginine hydrochloride produces a similar result when mixed with virus solutions (BAWDEN and PIRIE 1940).

There is one important difference between the insoluble complexes that have yet been produced artificially with the purified virus and the inclusions formed in the plant. We have already seen that the inclusions are true crystals with a perfect three-dimensional regularity,

but the needles produced by the precipitated virus after purification are not true crystals (BERNAL and FANKUCHEN 1937). These distinctions will be discussed in greater detail later, but there is evidence that some of the processes of purification, for example, precipitation with acid or salts, increases the size of the particles of tobacco mosaic virus, the particles apparently aggregating linearly to form rods. It is possible that the absence of a three-dimensional regularity in the precipitates of purified virus is a result of this alteration in size and shape, the elongated particles being unable to arrange themselves in a regular crystalline lattice.

At first sight the X-body, because of its amorphous nature, is much more difficult to connect with the virus. However, the formation of crystals, indistinguishable in appearance and behaviour from the crystalline inclusions, within the degenerating bodies of aucuba mosaic strongly suggests that the two kinds of inclusion have a common constituent (Fig. 4). Also, there is now considerable direct evidence that the X-bodies contain virus. LIVINGSTONE and DUGGAR (1934) found that the protoplasmic contents of the cell contained more virus than the sap, while SHEFFIELD (1939) has actually isolated individual X-bodies and shown them to be infective. She removed the bodies from infected cells by means of fine micro-pipettes, washed them thoroughly in buffer solutions, and then suspended them in water before inoculating them to *N. glutinosa*. From a consideration of the amount of purified virus necessary to give infections it is apparent that only few lesions could be expected from a single body. Depending on the host plant and method of inoculation from 10^{-8} to 10^{-10} gms of purified aucuba mosaic virus are required to give infection. If the bodies are assumed to be spherical with an average radius of 10μ , then they will have a volume of 4×10^{-9} c.c. Within the accuracy required for the comparison the bodies can be taken as solid with a specific gravity of 1. Then the weight of a single body will be approximately 4×10^{-9} gms, which is of the same order as the weight of purified virus necessary to give infection. SHEFFIELD suspended lots of 10 isolated X-bodies in water and compared their infectivity with solutions containing a known weight of purified aucuba mosaic virus. The results obtained are summarised in Table 1.

Table 1:
Aucuba mosaic virus. Infectivity of isolated inclusions compared with purified virus.

Inoculum	Concentration	No. of leaves inoc.	Total No. of lesions
Water	—	66	0
X-bodies	10 in 4 c.c.	66	16
Purified virus	10^{-8} gms per c.c.	66	20
Water	—	6	0
X-bodies	10 in 0.8 c.c.	6	7
Purified virus	2×10^{-7} gms per c.c.	6	4

However, the demonstration that the isolated and washed X-bodies from plants with aucuba mosaic are infective cannot be taken as proof that the virus is an essential part of the body. For it has been shown that the bodies also frequently contain chondriosomes and oil globules, which are presumably absorbed during the formation of the body, and the virus may have been acquired in a similar manner. But as the infectivity of the isolated X-bodies is, weight for weight, of the same order as the purified virus, it seems probable that their virus content is too great to be explained merely by the accidental absorption of virus.

Unfortunately, there is no critical test for the presence of small quantities of virus other than demonstrating infectivity. Purified preparations of the tobacco mosaic viruses consist of nucleoproteins, differing from the nucleoproteins characteristic of nuclei in that the nucleic acid contains ribose instead of a desoxy pentose (BAWDEN and PRIE 1937*a*). Feulgen's reagent readily identifies desoxy pentose, but there is no such colour test for detecting nucleic acids of the ribose type. The effect of staining with Feulgen's reagent in differentiating between the nucleus and X-body has already been described. It is apparent therefore that the bodies do not contain a desoxy pentose type of nucleic acid, but at present there is no method of determining whether they contain the nucleic acid characteristic of the virus.

If the X-bodies are composed largely of an insoluble complex formed by the union of the virus with a constituent or constituents of the host plant, the complex must obviously differ considerably from that suggested for the crystalline inclusions. The greater stability of the X-bodies, their amorphous nature and the fact that wide changes in hydrogen ion concentration do not affect them, all suggest a much firmer union, and that the ratio of virus to host constituents is less than in the crystalline inclusions.

Insoluble virus-protein complexes, probably containing less than 50% of virus and superficially resembling the X-bodies, can be formed *in vitro* by mixing preparations of tobacco mosaic viruses with their antisera. Amorphous precipitates rapidly settle out from such mixtures. These are not only more stable than the complexes formed by the union of the viruses with clupein, but are also unaffected by acid and are insoluble in strong salt solutions. In virus-antiserum mixtures kept circulating by convection currents, a sequence is obtained closely resembling that in cells infected with aucuba mosaic virus during the formation of X-bodies. Small particles first appear throughout the fluid. These fuse on coming together so that large aggregates are formed, which settle out at the bottom of the tube. We have seen that the X-bodies tend to be formed relatively early during infection when virus content is low and to disappear later with increasing virus content. If the bodies are composed of a virus-host complex with solubility relationships similar to those of the virus-antiserum precipitates, this behaviour can also be explained. For, although these precipitates cannot be dissolved by small changes in the pH or the salt content, their formation is greatly inhibited by the presence of too much virus and they dissolve fairly readily

in the presence of a great excess of virus (BAWDEN and SHEFFIELD 1939).

The similarities between the X-bodies and the virus-antibody complexes have been described here merely to show that phenomena in the plant can at least be simulated in part *in vitro*. It is not intended to suggest that the inclusions are actually complexes formed by the union of virus and antibody, for there is no valid evidence that plants contain or produce antibodies. It is possible, however, that they contain proteins capable of uniting with the virus and rendering it insoluble. Any such combination might also be expected to act in part as a protective mechanism, for it is difficult to imagine any virus that might be rendered insoluble in the inclusions possessing great biological activity.

Intracellular inclusions in infections with viruses other than tobacco mosaic viruses:— It is impossible to give a complete list of the viruses which do and which do not cause the production of intracellular inclusions in infected plants. In many published accounts of symptoms no mention is made of their occurrence but there is no indication as to whether they have been sought. Also, the contents of uninfected cells are extremely varied. In addition to the nucleus, cytoplasm and cell sap, the living cell contains plastids, chondriosomes, oil globules, crystals of calcium oxalate, and numerous particles of unknown composition, many of which are birefringent. Unless adequate comparisons are made between healthy and infected plants, therefore, it is possible for normal constituents of cells to be mistaken for virus inclusion bodies. This is especially so as infection often increases both the rate of cytoplasmic streaming and the numbers of crystals and particles that can be found in healthy plants. Most normal cells contain only one nucleus, but it is not uncommon for those of virus-infected plants to contain two (SHEFFIELD 1936; SALAMAN 1938). Hence it is necessary that a clear distinction should be drawn between the nucleus and any suspected inclusion bodies.

In Table 2 are listed the viruses which have been found to cause undoubted inclusions, and those causing diseases which have been carefully examined for inclusions and none found.

The viruses listed are only a few of those recognised, but the majority have not been investigated sufficiently to warrant including. Both lists contain viruses with widely different properties *in vitro*, and it is impossible with our present knowledge to indicate any features which might determine the production of inclusions. The properties of cucumber viruses 3 and 4 approximate much more nearly to those of tobacco mosaic virus than to those of the other viruses included in the second list, and *in vitro* these viruses precipitate in the same manner and in similar conditions (BAWDEN and PIRIE 1937*b*). Here there may be an effect of the host plant in inhibiting the formation of inclusions. Only members of the cucurbitaceae have been infected with cucumber viruses 3 and 4, and as these are immune to tobacco mosaic virus, there are no common hosts in which the behaviour of the two types of virus can be compared. Expressed sap of cucumber plants is considerably more alkaline than tobacco sap, pH about 7.8

Table 2:

<i>Viruses known to cause the formation of intracellular inclusions.</i>	<i>Viruses which apparently do not cause the formation of intracellular inclusions.</i>
Dahlia mosaic	Cucumber 1
Dwarf (Stunt) of rice	Cucumber 3 and 4
Hippeastrum mosaic	Potato leaf roll
Hyoscyamus 3	Potato paracrinkle
Iris stripe	Tomato spotted wilt
Oat mosaic	Tomato big-bud
Pea 2	
Phaseolus 2	
Potato "X" (many strains)	
Potato "Y"	
Sandal-Spike	
Sugar beet curly top	
Sugar-cane Fiji disease	
Sugar-cane mosaic	
Tobacco mosaic (many strains)	
Tobacco ringspot	
Tobacco severe etch	
Tomato bushy stunt	
Wheat mosaic	
Wheat rosette	

instead of 5.8, and as this would render both the viruses and complexes of the type suggested more soluble, this difference may be responsible for the apparent absence of inclusions. Alternatively, cucumbers may be lacking in substances capable of uniting with and precipitating the viruses.

That cucumber is perhaps an unsuitable host for the formation of inclusions is indicated by work on tobacco ringspot virus. In tobacco this causes the production of large numbers of inclusions, but in cucumber, which is quite susceptible, only occasional and rather indefinite bodies are seen (WOODS 1933; BAWDEN and SHEFFIELD 1939). Tobacco ringspot virus resembles tobacco mosaic virus in that it produces both amorphous and crystalline inclusions. The former are amoeboid and vacuolate, closely resembling those of tobacco mosaic. The crystalline inclusions have been seen only in infected solanaceous plants. They occur with much less frequency than those in plants with tobacco mosaic and have a different crystalline form, most being rectangular blocks. They are biaxial crystals, for when seen between crossed Nicol prisms they are birefringent along all axes (Fig. 10). Crystals not seen in healthy plants of the same species have also been described in oats, millet, barley and maize suffering from mosaic (SOUKHOV and VOOK 1938). The commonest are small needles, but the epidermal cells contain large protein crystals, which sometimes break up into small aciculate crystals.

Although intranuclear inclusions have been described in about thirty different virus diseases of animals, there was no convincing demonstration of their occurrence in virus infected plants until KASANIS (1939) found that the nuclei of solanaceous plants suffering from severe etch invariably contain abnormal bodies (Fig. 11). These give the usual protein reactions and with most stains behave similarly

to the nucleoli. They are thin flat crystalline plates, birefringent when viewed edgewise. They are first seen about a week after external symptoms become obvious, and they occur in all tissues except the growing points. They vary in size and up to thirty may be produced in a single nucleus.

Similar inclusions are also produced in the nuclei of plants infected with mild etch virus (BAWDEN and KASSANIS 1941), but these are larger and fewer than in plants infected with severe etch virus. Not all the intranuclear inclusions formed by mild etch virus seem



FIG. 11.—Pith cells from *Nicotiana Tabacum* infected with severe etch virus, stained with haematoxylin. All the nuclei contain darkly stained, plate-like inclusions. $\times 500$. (Photograph by B. KASSANIS).

to be flat plates, for some show a characteristic cross suggesting that they are probably eight-sided bi-pyramids (Fig. 12). The intranuclear inclusions are all remarkably stable; they can be extracted from the nuclei, when their structure is unaffected by pH changes between 2 and 10 or by the addition of alcohol. Except for the presence of these crystals, the nuclei appear normal and they still contain nucleoli. SHEFFIELD (1941) found that infection with severe etch virus sometimes stimulated the nuclei of fully differentiated cells to divide, so that binucleate cells were produced. Any intranuclear crystals were then extruded into the cytoplasm, but as soon as the daughter nuclei were reconstituted further crystals appear in them.

MCWHORTER (1940) has found isometric crystals in nuclei and cytoplasm of leguminous plants infected with pea virus 2 and Phase-

olus virus 2. Similar crystals were also seen inside the nucleoli. Usually five or more occurred in a nucleolus, but sometimes one large crystal might fill it. They vary from 0.3μ to 4μ in diameter, and give the usual protein reactions. Like those in plants suffering from etch, these inclusions can be removed from the nucleus and remain stable in saline.

In infections with other viruses only the X-body type of cytoplasmic inclusion has been reported. The differences in morphology that have been described between inclusions formed by different viruses are about as great as those between the bodies formed by tobacco mosaic and aucuba mosaic viruses. Wherever microchemical tests have been made, positive tests for protein have been obtained and the inclusions have failed to stain with Feulgen's reagent. SHEFFIELD (1934, 1941) has shown that the X-bodies formed by Hyoscyamus virus



FIG. 12. — An epidermal cell from a tobacco plant infected with mild etch virus. The intranuclear inclusion shows a definite cross, suggesting that the crystal is a bipyramid. $\times 900$.

3 and severe etch virus are a result of the aggregation of small particles circulating in the cytoplasm; CLINCH (1932) and SALAMAN and HURST (1932) suggest that those produced by potato virus "X" are also aggregates.

Where the history of the inclusion body has been followed in detail, it has been found to have a relatively short existence, usually changing in appearance before disintegrating and disappearing. Disintegrating bodies in plants infected with Hyoscyamus virus 3 often contain long, thin, crystal-like needles, resembling those formed by precipitates of tobacco mosaic virus. They show no extinctions in the polarising microscope, but whether this is because of their small size or because they are really isotropic is unknown. Old inclusions in severe etch plants may also crystallise, especially in leaves that become deformed. The usual form is again needle-like, varying from 1μ to 10μ long (Fig. 13), but these are birefringent; birefringent particles of other shapes are also formed. Whether these are true crystals or paracrystalline fibres is unknown. They can sometimes be seen in malformed leaves within a month of inoculating young seed-

lings, but they usually need longer than this to develop in any quantity. All the bodies in a malformed leaf may not contain crystals, and some parts of a single large body may have them while other parts remain granular and isotropic. These needles are less stable than the intranuclear inclusions; they dissolve and the X-body regains its earlier appearance when treated with weak acid. SHEFFIELD (1941) has extracted these cytoplasmic inclusions and shown that they are infective. They differ from the inclusions of aucuba mosaic in that they can be pricked with a micro-needle and even cut into pieces without disintegrating. They are chemically complex, for they contain fats and oil globules as well as proteins.

No appreciable differences have been found between the properties of severe etch virus, Hyoscyamus virus 3 and potato virus Y *in vitro*, yet they differ widely in their behaviour in the plant.

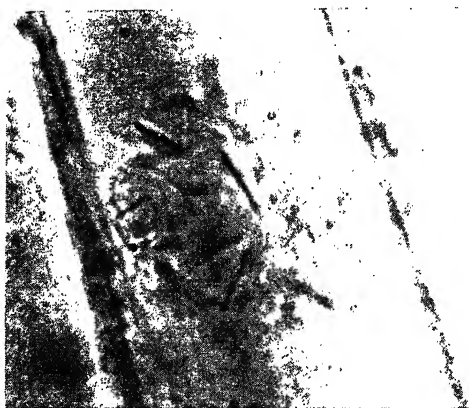


FIG. 13.—An epidermal cell from a malformed leaf of a tobacco plant which was infected with severe etch virus when a young seedling. The cytoplasmic inclusion body has crystallised and contains needles; these are birefringent when viewed in polarised light. $\times 900$.

Severe etch virus produces a large number of inclusions in both nuclei and cytoplasm and Hyoscyamus virus 3 produces many cytoplasmic inclusions but none in the nuclei. For long no inclusions were found in plants infected with virus Y, and now only a few, diffuse ones have been seen. If the inclusion bodies are insoluble complexes of viruses and host constituents, this suggests that the substances with which the viruses unite are not normal host constituents but products specific to the particular infection, for the three viruses infect the same plants and precipitate in such similar conditions *in vitro* that any normal host constituent precipitating with one would also be expected to precipitate with the others.

Although X-bodies have been found in all tissues except the apical meristem, individual viruses produce them with greater readiness in different tissues. Those of tobacco mosaic are most common in the epidermal layers of the leaf, with potato virus "X" the palisade cells contain the greater number, and in sugar beet suffering from curly top they are restricted to the phloem and adjacent tissues.

By contrast, *Hyoscyamus virus 3* appears to produce them equally in all tissues. The majority of workers state that the inclusions are associated with external symptoms, and BEALE (1937) found that tobacco plants infected with a "masked" strain of tobacco mosaic virus contained inclusions only in the occasional areas showing symptoms. But this association would not seem to be essential, for bodies have frequently been pictured in the roots of infected plants, and these show no external symptoms. Also, SALAMAN (1938) states that tobacco plants infected with a masked strain of potato "X" contain more inclusion bodies than those infected with more virulent strains producing symptoms.

There are slight morphological differences between the inclusions formed by unrelated viruses, but these are no greater than those between the inclusions formed by strains of the same virus. It is reasonable to suppose, therefore, that the various bodies described will have a similar mode of formation. HARTZELL (1937) has recently pictured inclusions in plants suffering from peach and aster yellows which he describes as intracellular bodies. These are much smaller than those found in other virus diseases and, according to HARTZELL, are actively motile. They were also seen in infective insect vectors. However, these bodies are so ill-defined that until they are more clearly differentiated from the contents of uninfected cells, and their movement from Brownian movement, it would seem premature to regard them as virus inclusion bodies.

Internal changes other than intracellular inclusions:—The formation of intracellular inclusions depends more upon the infecting virus than upon the type of external symptoms produced. Two viruses, for example tobacco mosaic and cucumber 1, may cause very similar external symptoms, but inclusions will be formed only in plants infected with the former. Other types of internal changes in virus-diseased plants much more accurately reflect the external symptoms, and in general viruses causing similar external symptoms also cause similar histological changes.

The leaves of plants showing mosaic have been studied by a large number of workers. Usually both the size of the cells and their contents are altered. The chlorotic areas are thinner than the green areas, usually because of a reduction in the length of the palisade cells, and the intercellular spaces are smaller. The following measurements made by CLINCH (1932) on the potato variety President suffering from crinkle show this reduction:—

	Thickness of leaf	Length of palisade cells
Yellow area of crinkled leaf. . . .	113 μ	45 μ
Dark green area of crinkled leaf. . . .	137 μ 148 μ	57 μ 72 μ
Healthy leaf	140 μ 147 μ	52 μ 56 μ

The chloroplasts are smaller than usual, more granular, and less deeply coloured, and often have irregular outlines. The whole effect is one of reducing the assimilatory tissues. How this reduction is brought about is uncertain. The view has frequently been put forward (COOK 1930; SHEFFIELD 1933) that the viruses have no destructive effect on chlorophyll or plastids, but act only by inhibiting plastid formation. This is largely based on the fact that systemic symptoms are always most definite on leaves which are developing at the time of infection, mature leaves often being quite unaffected. It seems that some viruses at least, especially tomato stripe in tobacco (SMITH 1935), must have an effect on mature chloroplasts, for if fully mature leaves are rubbed with a concentrated virus preparation they may become completely chlorotic. Mature leaves which are not inoculated rarely develop a high concentration of virus. Thus it is probable that the absence of symptoms in leaves mature when plants are infected can be explained on the basis of their low virus content rather than on the inability of the virus to affect mature plastids.

Although the assimilatory tissues are reduced, there is often an abnormal accumulation of starch in cells of chlorotic areas. This condition is found in many diseases of the yellows type and is especially definite in potato leaf-roll, where the chloroplasts are frequently so full of starch that they may burst (CLINCH 1932). In this disease and in many others, such as sereh disease of sugar cane, curly top of sugar beet, raspberry curl and aster yellows, this excessive accumulation of carbohydrates is accompanied by abnormalities in the phloem. In the potato the sieve tubes and companion cells of affected plants become necrotic (QUANJER 1913). QUANJER (1931) considers that the death of the phloem elements is responsible for the accumulation of carbohydrate in the leaf, but MURPHY (1923) and BAWDEN (1932) state that rolled leaves containing much starch are often found in the first year of infection when there is little or no necrosis of the phloem. In sugar beet with curly top there is a similar necrosis of the phloem elements but in addition the pericycle may also turn necrotic. Near the degenerate areas there is often hypertrophy and hyperplasia, leading to a thickening or distortion of the veins and to a formation of protuberances (ESAU 1933). According to ARTSCHWAGER and STARRETT (1936) the nuclei of sugar beet cambium are also affected. Early in infection there is an increase in nucleolar and chromatin material, the nuclei enlarging and becoming misshapen. Later, the hypertrophied nuclei break down liberating fragments which remain unaltered in the cytoplasm.

Potatoes suffering from top-necrosis also show a degeneration of the phloem elements in leaves, stems and tubers. The necroses always arise in the phloem, but once originated spread rapidly to all other tissues. Large intercellular spaces may be formed which are filled with gum-like deposits. The cell walls become thickened, because of the deposition of lignin or suberin, while the cell contents either disappear or are changed into darkly staining substances rich in pectin. Phellogens are frequently formed around necrotic areas,

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Chapter IV

TRANSMISSION

Transmission by grafting:—Before a disease can be recognised as a virus disease it must be shown to be infectious, that is, the cause must be transmitted to healthy plants and the diseased condition reproduced in them. There are three main methods of transmitting viruses; by grafting healthy plants with scions taken from diseased ones, by inoculating healthy plants with sap expressed from diseased ones, and by insects feeding on healthy plants after having fed on diseased ones. Some viruses, for example, tomato spotted wilt and cucumber 1, can be transmitted by all three methods. Others, as far as is known, can be transmitted by two of the methods and the remainder by only one. Potato virus "X" and cucumber virus 3 are transmitted by grafting and by inoculation, but have not been transmitted by insects. Those causing curly top of sugar beet and potato leaf roll are transmitted by grafting and by insects but not by inoculation, whereas others, such as those causing potato paracrinkle, peach rosette, hop nettle-head and tomato big-bud, have been transmitted only by grafting. It is probably that many viruses of the last type are transmitted by some other means, probably by insects, and further work will no doubt greatly reduce the large number for which now the only known method of transmission is by grafting.

Transmission by grafting will be seen from the few examples given to be the most universal method of virus transmission. All viruses can be transmitted by grafting between plants in which they cause systemic infection, for once organic union is established between scion and stock transmission by grafting is merely a sequel to their ability to spread through vegetative parts of plants.

Systemic infection can also often be obtained in some hosts by grafting when inoculation gives only local lesions. For example, if potato virus "B" is inoculated to many potato varieties the only results are black necrotic local lesions, whereas if such varieties are grafted with scions from infected carriers they die with top-necrosis. Similarly, *N. glutinosa*, if grafted with a tomato scion infected with tobacco mosaic virus, dies with a systemic necrotic disease closely resembling top-necrosis. In some diseases, viruses are not found in all parts of the affected hosts: then transmission by grafting will only occur when parts of the hosts containing virus are used as scions. No transmission of phony-disease of peach takes place if the aerial portions are used as scions, but root grafting is successful. Tobacco necrosis viruses give only local lesions in leaves, but apparently become systemic in roots. This virus has not yet been transmitted by grafting, but it is probable that root-grafting would again be successful.

Transmission by grafting is so characteristic of viruses that, taken together with the absence of a visible parasite, it has almost become the criterion as to whether or not a plant disease shall be placed in the virus group. The methods of grafting are those used in ordinary horticultural practices, but are varied slightly with the type of tissue to be grafted. With herbaceous plants the most suitable is the cleft graft. The top of the healthy plant to be grafted is cut off, preferably through or immediately above a node, and the stem cut down the middle for an inch or so. An apical shoot is taken from the diseased plant, trimmed to a few small leaves, and the stem cut to a wedge-shape. The diseased wedge is inserted into the cut stem, bound around lightly, and the plant kept in a moist, warm atmosphere for a few days, until a firm union has been established. In these conditions rapidly growing side shoots are produced from the stock, an ideal state for the development of good symptoms. A further advantage of this method is that the top of the grafted plant which has been cut off can often be rooted and kept as a control.

With woody plants or trees, budding may be preferable to grafting. A bud is excised from the diseased plant so that it carries cortical tissues and phloem; two slits at right angles to one another are then made in the stem to be budded reaching to the cambium, and the bud is inserted in the opening so that the two cambial tissues are in contact.

The presence of a bud in the piece of diseased tissue to be grafted is advisable, but not essential. SREENIVASAYA (1930) has shown that spike-disease of sandal, which is not transmitted by inoculation, can be transmitted merely by inserting pieces of infected tissue between the wood and the bark of a healthy stock, callus formation apparently being sufficient to unite the infected and healthy tissues. Grafts with buds, however, were more reliable and produced symptoms quicker.

For working with strawberry runners, HARRIS (1932) has used a method of inarching with success. Equal areas of both the scion and stock-stolons have their epidermis and cortex removed. Oblique cuts are made in both stolons, and the pieces interlocked so that two cut surfaces of one stolon are in contact with cut surfaces of the other. The junction is then bound. The binding of the grafts can be done with raffia (bast) or with thin rubber tape. Alternatively, the grafts can be sealed with grafting wax. In the potato successful virus transmissions can often be obtained by means of tuber grafts. This method has the advantage over the grafting of growing plants that the grafts can be made during the winter, so that the treated plants show symptoms as soon as they come above ground. The method most used is known as core grafting. A core is removed from the healthy tuber by means of a cork borer. This core should have an eye so that it can be planted to act as a control. Using a slightly larger cork borer a core is removed from the infected tuber and inserted into the hole made in the healthy tuber (MURPHY and MCKAY 1926).

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(1941). This is the use of the parasite dodder to link-up diseased and healthy plants. The method is not as certain as ordinary grafting, because all viruses are not transmitted by dodder. Aster yellows, bushy stunt, curly top, pea mottle and tobacco mosaic viruses were transmitted but tobacco ringspot and pea wilt viruses were not. It is unlikely that this method of transmission is common in nature, but it may have experimental application in testing suspected virus diseases in species difficult to graft, and in transmitting viruses between hosts that cannot be intergrafted.

Transmission by mechanical methods: — Grafting is the only method whereby many viruses are known to be transmitted and it is extensively used for the recognition of virus diseases, but it is obviously of no value for studying the properties of viruses *in vitro*. Hence, only those viruses that are transmitted by additional methods, especially mechanical methods, have been studied in any detail. The principle of these methods of inoculation consists of introducing infective sap into wounds made in healthy plants. In the early work on viruses, injections were made with needles or pins dipped in the sap, or sap was placed on the leaves and scratches were made through it. Although these methods were reasonably successful with some viruses, it soon became evident that the excessive wounding they produced was a disadvantage. At the present time it is generally accepted that wounds are necessary for the entry of viruses into plants, but that the less the host plant is damaged in the production of entry points the more efficient is the method for producing infections.

For the simple transfer of virus from one plant to the other by inoculation of sap, the following method is widely used. Leaves from the infected plant are ground up with a pestle and mortar, and the extracted sap then rubbed on to leaves of the healthy plant with the pestle. For transmission in local lesion experiments the methods need refining, and it has been shown in Chapter 2 that the various techniques employed are all designed to distribute the inoculum evenly over the leaves with a gentle rubbing that produces no visible damage. Most of the methods have been designed to break the hairs without damaging the leaf tissues, for it has been generally believed that the broken hair cells formed the most suitable entry points. But BOYLE and MCKINNEY (1938) have recently produced evidence suggesting that the hairs may not play such an important part as was previously believed. They found that the number of infections with tobacco mosaic virus was not dependent upon the numbers of trichomes, and that in pepper plants there was no significant difference between the number per unit area on areas with and without hairs. As they were able to produce only few infections by inoculation methods which broke only the trichomes, they suggest that bruising the epidermal cells without causing excessive damage is more important than breaking hair cells and that most infections occur in the other epidermal cells.

This suggestion may explain the somewhat curious results obtained with some viruses which, although difficult to transmit mechanically by needle-scratch or by rubbing, are readily transmitted by rubbing

if powdered carborundum crystals are added to the inoculum. Using this technique RAWLINS and TOMPKINS (1936) were able to get large numbers of infections with tomato spotted wilt virus from lettuce, and with other viruses affecting beans, celery and cabbages, which gave few or no infections by the ordinary rubbing method. The small carborundum crystals pierce the epidermal cells but apparently do not injure them sufficiently to prevent the viruses from multiplying. Potato virus "A" gives somewhat similar effects. This is readily transmitted to tobacco plants by ordinary rubbing but is not so transmitted to potato plants (CLINCH and LOUGHNANE 1933). However, if carborundum powder is added to the inoculum the virus can be readily transmitted to both kinds of plant. As the leaves of both plants have numerous trichomes that are broken by simple rubbing, it seems that the difference probably lies in the other epidermal cells. In the tobacco it is possible that rubbing on its own affects the epidermal cells sufficiently to permit the entry of virus "A", without damaging them enough to prevent virus multiplication. The potato has a tougher leaf and, in the absence of carborundum, it is possible that rubbing or scratching sufficient to make an entry point also damages the epidermal cells too much to permit the virus to multiply. Other viruses are readily transmitted to potatoes by rubbing or needle-scratch. It may be that these enter in a different manner, perhaps through the trichomes, but the facts that they occur in a greater concentration in infective sap and are more stable *in vitro* suggest that they may be able to enter and multiply in cells too damaged to permit the multiplication of virus "A" (BAWDEN 1936).

An entry point suitable for one virus is not necessarily suitable for another. When tobacco mosaic virus is rubbed over all the leaves of a well-grown *Nicotiana glutinosa* plant there is a definite trend of decreasing susceptibility from the bottom to the top. With tomato bushy stunt virus there is also a susceptibility gradient, but the other way around. Thus when preparations of the two viruses are rubbed onto opposite halves of *Nicotiana glutinosa* leaves, the bushy stunt virus will produce more lesions than tobacco mosaic virus on the upper leaves while it will produce few or none on the lower leaves, which will bear large numbers of tobacco mosaic virus lesions. As rubbing will have broken similar numbers of trichomes and bruised the epidermal cells equally with both viruses, it seems that something more than wounding is needed to produce suitable entry points. The injured cells with which the viruses come into contact must also be in a condition to receive infection, and this condition differs for different viruses. Even with plants that can become systemically infected by inoculation, differences in the ease with which this can be done varies with the host. Tomato plants, when not more than about six inches high, are almost killed within a few days of inoculation with bushy stunt virus. Plants a little larger become systemically infected on inoculation, but react only with slight symptoms and have a low virus content, and still larger plants do not become systemically infected at all by inoculation.

The view that wounds are necessary for the entry points of viruses into plants has been questioned on several occasions. DUGGAR and

JOHNSON (1933) obtained infections with tobacco mosaic virus by spraying healthy plants with infective sap through a fine atomiser, and concluded that the virus was entering through the stomata. SMITH and BALD (1935) also obtained infections with tobacco necrosis virus merely by spraying infective sap over leaves believed to be undamaged, and SMITH (1935) states that large numbers of local lesions were produced on tobacco seedlings when they were sprayed with suspensions of potato virus "X". On the other hand, CALDWELL (1931, 1932) states that he has injected the intercellular spaces of *Nicotiana glutinosa* with extracts of tomato aucuba mosaic virus and never obtained infections unless cells were punctured, and that the exudates from hydathodes of infected tomato plants do not contain virus. Similarly, JOHNSON (1936, 1937) failed to demonstrate stomatal infection of a highly susceptible tobacco hybrid by spraying with tobacco mosaic virus, and SHEFFIELD (1936) obtained no infections when uninjured *Nicotiana glutinosa* were sprayed with aucuba mosaic virus. PRICE (1938) has repeated spraying experiments with tobacco necrosis on a number of different hosts. After they were sprayed, the plants were divided into two lots. The leaves of one lot were rubbed while the others were untouched. The leaves of the rubbed plants developed some hundreds of lesions, whereas the majority of those unrubbed remained quite healthy while the others developed only a few lesions. Significantly more lesions developed on unrubbed tobacco than on other plants. PRICE suggests that this is not due to the greater susceptibility of tobacco plants to stomatal infection, but to the fact that these have the most compact type of growth and their leaves therefore have a much greater chance of rubbing together during natural growth movements. When tobacco plants were trimmed so that their leaves no longer had any opportunity of rubbing together, no lesions developed on sprayed plants.

With such conflicting evidence the possible occurrence of stomatal infection in the absence of wounds cannot be ruled out. But if it occurs it must be seldom, and it seems that for all practical purposes it can be ignored.

Although mechanical transmission is of greatest importance in experimental work, it is by no means a negligible factor in the spread of virus diseases in nature. Some of the viruses occurring most commonly, such as tobacco mosaic and potato "X", do not appear to be insect transmitted. These are transmitted extremely easily by inoculation methods and it is highly probable that they are transmitted in nature by mechanical means. Tobacco mosaic virus is so stable that it is not completely inactivated by the processes of curing tobacco. Its introduction into growing crops of tobacco and tomato is largely a result of workmen handling the plants after using infected tobacco, or of growing the plants in infected soil. Once established, it spreads rapidly by the rubbing together of diseased and healthy plants and by cultural operations involving the repeated handling of plants. LOUGHNANE and MURPHY (1938) have recently shown that potato viruses "X" and "F" also spread readily between plants whose haulms are in contact, the rate of spread being increased when the rubbing of infected and healthy leaves is increased by allowing a fan

to play on them. Although this method of transmission may explain the spread of these two potato viruses within a crop, some other method of transmission from a distance would seem necessary for the production of the first source of infection, unless it can be attributed to the presence of infected "groundkeepers" or rogues. Potato spindle tuber is transmitted between tubers by the knife used for cutting the seed sets and it is possible that potato virus "X" would also be carried in this way.

The possibility of the transmission of viruses below ground-level by the infection of roots has been frequently raised. There is a good deal of evidence that it does occur, but again the results of different workers are conflicting. That the soil is a common source of primary infection for tobacco mosaic virus has been shown repeatedly (McKINNEY 1927; LEHMAN 1934; JOHNSON 1937). It has usually been assumed that infection from the soil takes place through the roots, although this has not been satisfactorily demonstrated and various workers have found it difficult to infect plants by inoculating roots. JOHNSON (1937) states that infection through the roots does not take place commonly even if the roots are deliberately wounded. He considers that infection of plants from infected soil takes place only through contact between the leaves or stems and the soil. Some recent work would also suggest that if root infection occurs it is probably not of great economic importance.

SMITH (1937*a*, 1937*b*) has found that the roots of tobacco plants grown in soil contaminated with tobacco necrosis virus become infected but the tops, except for occasional leaves touching the soil, remain free from virus. PRICE (1938) has confirmed this. He has also found that tobacco and *Nicotiana glutinosa* grown in soil contaminated with tobacco mosaic virus behave similarly. Many of the roots of such plants become infected. These, when ground up and inoculated to leaves of *Nicotiana glutinosa*, produce large numbers of lesions, but the aerial portions show no symptoms and remain free from virus. Roots grown in water culture solutions heavily contaminated with virus do not become infected unless they are rubbed with the virus. The virus is therefore unable to enter uninjured roots and presumably enters those grown in soil or sand through abrasions made by the growth movements of the roots. This work provides a definite demonstration of root infection, but the most interesting and surprising result is the limitation of infection to the roots. With tobacco necrosis virus, or with tobacco mosaic virus in *Nicotiana glutinosa*, this is not so surprising, for when leaves are inoculated with these viruses only local lesions are produced. But the infection of a tobacco leaf with tobacco mosaic virus is followed by the spread of the virus to all parts of the plant including the roots. It seems, therefore, that although the virus is able to move freely from the stem down into the roots, it is unable to move from the roots into the stem. Unfortunately, PRICE kept his plants for only three weeks and it is therefore not possible to say whether the virus would ultimately pass into the tops, but the results support the statement of JOHNSON (1937) that root infection is of little economic importance.

At Rothamsted we have grown tobacco and tomato plants in soil contaminated with tobacco mosaic virus and kept such plants for months. Often the roots of these plants were highly infective, giving hundreds of lesions per leaf when ground up and tested on *N. glutinosa*, although the leaves looked healthy and were virus-free. Occasionally, however, individual plants kept for long periods did develop symptoms in the tops. We attempted to influence this by cutting off the tops to force the growth of new side shoots and by putting such plants in the dark for some days to prevent the flow of food-stuffs from the leaves to the roots. Both treatments slightly increased the number of plants that became infected, but neither could be relied upon to produce infection in the tops. FULTON (1941) infected the roots of tobacco and tomato plants with several different viruses by direct inoculation, but with no virus did he get any infection of aerial parts. The downward movement from the point of inoculation was as rapid as when stems were inoculated, but the upward movement in the roots was slow and restricted. If these results apply to other viruses, it seems probable that the transmissions of narcissus stripe (McWHORTER 1932) and potato mosaic (McKAY and DYKSTRA 1932) which have been considered a result of root inoculation during cultural practices actually resulted from inoculation of the tops.

SMITH (1937*b* and *c*) has found that if he atomised suspensions of tobacco necrosis virus above tobacco plants the roots become infected. He also reports frequent natural spread among the plants in his glasshouses when growing in conditions usually regarded as safeguarded against accidental virus infections. To account for these facts he suggests that the virus is air-borne, implying that it is normally carried in the air, from which it falls on to the soil and then enters the roots. SMITH's results show clearly that plant roots become infected with this virus when the soil is watered with infective suspensions, and they have been confirmed by PRICE (1938). It is, therefore, not surprising that infections should also be obtained when the air above the soil is sprayed with suspensions of the virus, for the spray will naturally settle on to the soil and the final result will be equivalent to adding a small volume directly to the soil. The successful demonstration that a virus is air-borne requires more than this. It is necessary to show not only that virus deliberately placed in the air can infect a plant, but that the virus can actually get into the air. There is at present no evidence for this either with tobacco necrosis or any other virus. PRICE observed no natural infections with tobacco necrosis in glasshouses containing large numbers of infected plants. Infected leaves might, of course, be blown from one plant on to the soil in the neighbourhood of another, and it is possible that infections could arise in this manner. It seems probable that the natural infections with this virus are a result of growing plants in infected soils, of using contaminated water, or of the accidental mixture of infective plant debris with the soil. This is especially so as the natural infections are confined to the roots and to occasional leaves touching the soil, for if the virus were present in the air it would not all be expected to settle on to the soil. Some would

fall on the leaves and the mere rubbing of healthy leaves would be expected to produce lesions, but this does not happen.

In the Rothamsted glasshouses natural infections occur, but these are often caused by viruses or virus-strains distinct from those being worked with in large quantities. They also happen as frequently in houses containing no artificially infected plants as in those containing large numbers. Hence it seems improbable that artificially infected leaves supply the sources of many of these natural root infections, and more likely that some soil factor is responsible.

To summarise:—Many viruses have not yet been transmitted by mechanical methods of inoculation. Of those that have, some are readily transmitted, some only with difficulty and in special conditions, and others can be inoculated to some hosts susceptible when grafted but not to all. Inoculation to some hosts may also produce symptoms different from those produced by grafting. The evidence available suggests that viruses cannot enter uninjured plants, but that for infection to occur the wounding supplying the entry points should cause the minimum of damage to the host tissues.

Transmission by insects:—In nature the infections with most viruses are undoubtedly the result of insect activity. The insect vectors pick up the viruses while feeding on infected plants and later transmit them to other plants on which they feed. Most efficient vectors feed by sucking rather than biting, and there are only few authentic records of insects with biting mouthparts acting as vectors. This may in part be explained by the greater damage inflicted on the host by biting insects, and by the fact that biting insects do not inject saliva into plants while they are feeding. But there are probably additional and more specific reasons, for there seem to be fairly close relationships between many viruses and their vectors. Many viruses are transmitted by one or a few closely related species, but not by others, although these may have similar feeding habits and be vectors of other viruses. There are exceptions to this specificity of which the most striking is potato spindle tuber virus. This is transmitted by aphids (SCHULTZ and FOLSOM 1925), grasshoppers, the tarnished plant bug, flea beetles, the leaf beetle and the larvae of Colorado beetle (Goss 1931). The virus causing mosaic of squash plants has also been transmitted by beetles and aphids (FREITAG 1941), sugar cane mosaic by aphids and a leaf hopper (INGRAM and SUMMERS 1938) and onion yellow dwarf by more than 50 different aphids (DRAKE, HARRIS and TATE 1933). An unusually high degree of specificity has been described with some viruses, related strains of which are transmitted specifically by different vectors. Celery yellows virus, for example, is transmitted by *Thamnotettix montanus* and *T. geminatus* whereas the related strain causing aster yellows is not (SEVERIN 1934), but both strains have a common vector in *Cicadula sexnotata*. The related strains of potato yellow dwarf virus, however, appear to have quite distinct vectors, for BLACK (1941) states that the New York strain of this virus is transmitted only by *Aceratagallia sanguinolenta*, and the New Jersey strain only by

Agallia constricta. In Table 3 are listed the most important insect vectors together with the viruses they are known to transmit.

New vectors for viruses are being discovered regularly and there is no doubt that many viruses which up to now have been transmitted experimentally only by grafting and inoculation do have vectors. How difficult it can be to discover a vector is illustrated by the work on peach yellows. For years numerous insects were tested as vectors without any success, until in 1933 KUNKEL found that it was transmitted by a leaf-hopper, *Macropsis trimaculata*, which has only one brood a year and is active for only one month during the year. Again successful inoculation by vectors may only be possible when certain restricted conditions are fulfilled. The white fly that transmit cassava mosaic can live successfully on old leaves, but they are only able to transmit infection to young leaves, less than about one quarter grown (STOREY and NICHOLS 1938). Similarly *Myzus persicae* is able to transmit potato virus "F", the cause of tuber blotch, from potato plants only if these are also infected with virus "A" (CLINCH, LOUGHNANE and MURPHY 1936). Because of such difficulties in demonstrating experimentally that an insect is a vector, it is often assumed that all plant viruses are insect transmitted, but this is by no means certain. Tobacco mosaic virus and potato virus "X" occur in nature as commonly as any other viruses, and if they were insect transmitted it is to be expected that their vectors would be commonly occurring insects. Yet none of the numerous insects tested has been found to act as a vector, and it becomes more and more probable that such viruses are not transmitted by insects.

Table 3:
INSECT VECTORS.

Insects.	Viruses transmitted.
<i>Thrips:</i>	
<i>Frankliniella insularis</i> Frankl.	Tomato spotted wilt
<i>Thrips tabaci</i> Lind.	Pineapple yellow spot
	Tomato spotted wilt
<i>Leaf-hoppers:</i>	
<i>Agallia constricta</i> Van Duzee	Potato yellow dwarf (New Jersey strain)
<i>Aceratagallia sanguinolenta</i> Prov.	" " (New York strain)
<i>Cicadula sexnotata</i> Fall.	Aster yellows
	Celery yellows
<i>Cicadulina mbila</i> Naude	Maize streak
	Maize mottle
<i>Delphax striatella</i> Fall.	Cereal mosaics
<i>Draeculacephala portola</i> Ball.	Sugar cane chlorotic streak
<i>Draeculacephala</i> sp. and	
<i>Cameocephala</i> sp.	Alfalfa dwarf
<i>Euscelis striatulus</i> Fall.	Cranberry false blossom
<i>Eutettix phycitis</i> Dis.	Little leaf of egg plant
<i>Eutettix tenellus</i> Baker	Sugar beet curly top
<i>Lygus pratensis</i> Linn.	Rape mosaic
	Potato spindle tuber
<i>Macropsis trimaculata</i> Fitch	Peach yellows
<i>Nephotettix apicalis</i> Motsch.	Rice dwarf (stunt)
<i>Peregrinus maidis</i> Ashm.	Maize stripe
	Corn mosaic
<i>Perkinsiella saccharicida</i> Kirk.	Sugar cane Fiji disease
<i>Piesma quadrata</i> Fieb.	Sugar beet leaf crinkle
<i>Thamnotettix argentata</i> Evans	Tobacco yellow dwarf
<i>Thamnotettix geminatus</i>	Celery yellows
<i>Thamnotettix montanus</i>	Celery yellows

Table 3 (cont.)
INSECT VECTORS.

Insects.	Viruses transmitted.
<i>White-fly:</i>	
<i>Bemisia gossypiperda</i> M. and L.	Cassava mosaic Cotton leaf curl Tobacco leaf curl Cassava brown streak
<i>Bemisia</i> sp.	
<i>Aphids:</i>	
<i>Amphorophora rubi</i> Kalt.	Onion yellow dwarf Raspberry green mosaic Raspberry yellow mosaic
<i>Amphorophora sensoriata</i> Mason	Raspberry green mosaic Raspberry yellow mosaic Plum pox (mosaic)
<i>Anuraphis padi</i> Linn.	Narcissus mosaic
<i>Anuraphis roseus</i> Baker	Tulip break
<i>Anuraphis tulipae</i> Boyer	Sugar beet yellows Tobacco etch
<i>Aphis fabae</i> Scop.	Bean mosaic Cauliflower mosaic Cucumber 1 Lily rosette Onion yellow dwarf Western celery mosaic
<i>Aphis maidis</i> Fitch.	Onion yellow dwarf Sugar cane mosaic
<i>Aphis rhamni</i> Boyer	Potato "A" Potato "Y" Soy bean mosaic Tobacco etch
<i>Aphis rumicis</i> Boyer	Bean mosaic Narcissus mosaic Onion yellow dwarf Pea mosaic Sugar beet mosaic Sugar beet yellows Soy bean mosaic Western celery mosaic
<i>Brevicoryne brassicae</i> Linn.	Bean mosaic Cabbage ring necrosis Cauliflower mosaic Onion yellow dwarf Turnip mosaic Western celery mosaic
<i>Capitophorus fragaefolii</i> Cockill.	Strawberry crinkle Strawberry witch's broom Strawberry yellow edge Sugar cane mosaic
<i>Hysteroneura setariae</i> Thos.	Bean mosaic Soy bean mosaic Cucumber 1 Iris stripe Narcissus mosaic Onion yellow dwarf Pea enation mosaic (Pea 1) Potato spindle tuber Potato leaf roll Potato "A" Potato "Y" Tobacco etch Tulip break
<i>Macrosiphum gei</i> Koch	

Table 3 (cont.)
INSECT VECTORS.

<i>Insects.</i>	<i>Viruses transmitted.</i>
<i>Aphids:</i>	
<i>Macrosiphum pisi</i> Kalt.	Bean mosaic Alfalfa mosaic Narcissus mosaic Onion yellow dwarf Pea enation mosaic (Pea 1) Pea mosaic Narcissus mosaic Cauliflower mosaic Cucumber 1 Hyoscyamus 3 Soy bean mosaic Potato "A" Potato "Y" Potato leaf roll Tobacco etch Narcissus mosaic Narcissus mosaic Potato leaf roll Potato "Y" Soy bean mosaic Bean mosaic Cabbage ring necrosis Cabbage ringspot Cauliflower mosaic Commelina mosaic Cucumber 1 Dahlia mosaic Hyoscyamus 3 Iris stripe Lettuce mosaic Onion yellow dwarf Potato "A" Potato "F" Potato "Y" Potato leaf roll Potato unmottled curly dwarf Potato spindle tuber Pea enation mosaic (Pea 1) Pea mosaic Sugar beet mosaic Soy bean mosaic Sugar beet yellows Tobacco etch Tulip break Turnip mosaic Western celery mosaic Cucumber 1 Potato leaf roll Soy bean mosaic Banana bunchy top Bean mosaic Cauliflower mosaic Onion yellow dwarf
<i>Macrosiphum rosae</i> Linn.	
<i>Myzus circumflexus</i> Buckton	
<i>Myzus cerasi</i> Fab.	
<i>Myzus convolvuli</i> Kalt.	
<i>Myzus ornatus</i> Laing.	
<i>Myzus persicae</i> Sulz.	
<i>Myzus pseudosolani</i> Theob.	
<i>Pentalonia nigronervosa</i> Coq.	
<i>Rhopalosiphum pseudobrassicae</i> Davies	
<i>Beetles:</i>	
<i>Diabrotica soror</i> Lec.	Squash mosaic Curcubit ring mosaic Squash mosaic Curcubit ring mosaic
<i>Diabrotica trivittata</i> Mann	

Table 3 (cont.)
INSECT VECTORS.

Insects.	Viruses transmitted.
<i>Beetles:</i>	
<i>Disomycha triangularis</i> Say.	Potato spindle tuber
<i>Epitrix cucumeris</i> Harris	Potato spindle tuber
<i>Leptinotarsa decemlineata</i> Say.	Potato spindle tuber
<i>Systema toeniata</i> Say.	Potato spindle tuber

It will be seen in Table 3 that some viruses have been transmitted by a number of different insects whereas others have been transmitted by only one or two. Often, of course, this specificity is only apparent, for other insects have not been tested as vectors. But with some viruses there seems to be a rigidly specific relationship between virus and vector, as insects closely related to the known vector are unable to transmit. The table also shows the predominance of members of the *Aphididae* as vectors. These transmit more viruses than any other group of insects and some individual species can transmit many apparently unrelated viruses. This is especially true of the species *Myzus persicae*. The importance of this aphid partly results from its extremely wide host range and from the fact that it is usually the first insect to be tested by virus workers as a possible vector. However, in addition it does seem to have a special affinity for viruses, for even when other insects are able to transmit a virus *M. persicae* often transmits much more efficiently. How insects transmit is still uncertain, but a detailed account of the work on the relationships between viruses and their vectors is given in the next chapter.

Many plants in nature are infected by more than one virus simultaneously and this can often be detected by attempting transmission to healthy plants by different methods. On grafting, all the viruses will pass across and the original condition will be reproduced in the stocks. If one of the viruses is transmitted by inoculation and the other not, inoculation will produce a result different from grafting. Similarly if one virus is transmitted by insects and the others not, or if the viruses are transmitted by different insects, the use of vectors will give a result different from grafting. For example, from President potato plants showing the complex disease crinkle, potato virus "A" would be obtained by the use of aphids and potato virus "X" by mechanical inoculation. JOHNSON (1942), by using dodder as a method of grafting, has shown that clover mosaic is a disease caused by a dual infection with pea mottle and pea wilt viruses, for only the mottle virus is transmitted in this way.

Seed transmission:— Using the word seed in the agricultural sense of anything that is planted, seed transmission of viruses is extremely important, but using it in its more accurate botanical sense, it becomes much less so. For viruses readily infect tubers, bulbs, corms, runners, and all other vegetative organs used for propagation, but it is rare for them to persist through the winter in the true seed. Crops of potatoes, tulips, strawberries, raspberries or other plants propagated vegetatively, therefore, tend to be more severely affected by virus diseases than those raised annually from true seed, for the

latter will usually give rise to virus-free plants even though the parent plants were infected. This fact has been repeatedly demonstrated with various viruses, but beans suffering from mosaic are a striking exception as seed transmission is a fairly regular occurrence (REDDICK and STEWART 1919). Not all the seeds set by diseased plants are infected, and in the same pod there may be infected and virus-free seeds. The length of time the parent plant has been affected partly determines the percentage of infected seeds, for those raised from infected seeds produce a higher percentage than those which become infected during the growing season (NELSON 1932; HARRISON 1935). A further peculiarity of bean mosaic virus, which has not been recorded with others, is transmission by pollen. REDDICK (1931) states that pollen from diseased plants carries the virus and in pollinating healthy plants infects them. By contrast, CALDWELL (1934) was unable to demonstrate any virus in the pollen of tomato plants suffering from aucuba mosaic; pollen from plants infected with sugar beet curly top virus (BENNETT and ESAU 1936) and with tobacco mosaic virus and potato virus "X" (GRATIA and MANIL 1936) has also been found to be virus-free.

Occasional transmission by seed has been reported in some diseases other than bean mosaic, especially in cucurbit and lettuce mosaics. The actual numbers of diseased plants arising in this manner are extremely small, but they are by no means unimportant, for they supply infection foci immediately the plants are above ground. The chances for rapid spread within a crop are, therefore, considerably greater than if the first infection has to arrive from outside the crop. HENDERSON (1931) found that tobacco ringspot virus was often transmitted through the seed of infected *Petunia* sp. but not through tobacco. VALLEAU (1941), on the other hand, working with six strains of this virus, found that they were all seed-transmitted. The amount of seed set by infected plants was much less than in healthy plants, and there was also considerable pollen sterility.

The seed from infected plants apparently can often contain virus, although the plants raised from the seed are healthy and virus-free. AINSWORTH (1933) found tobacco mosaic virus in the seed coats of tomato seeds taken from infected plants even after they had germinated. The young plants were all healthy but the testa obviously afforded a possible source of infection for them. SHEFFIELD (1941) found the characteristic intranuclear inclusions of tobacco etch virus in the testa of seeds. The other portions of the seed did not contain inclusions, and the seed gave no infected progeny. With this virus the immature seeds were infective but lost their infectivity as they ripened.

It is still unknown why seed transmission should occur so rarely with viruses able to establish themselves in all other parts of plants including the fruits. A number of explanations have been put forward, such as the anatomical isolation of the embryo, the inactivation of the viruses by adsorption on to the storage proteins or by processes of maturation, but none has been supported by sufficient experimental evidence to make it convincing.

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Chapter V

RELATIONSHIPS BETWEEN VIRUSES AND THEIR INSECT VECTORS

Suggestions have been made that insects infect plants with viruses in other ways than by feeding upon them, for example, by defaecation and mechanical damage, but these have not been confirmed. On the other hand, it has been shown conclusively that insects denied all contact with plants except by their mouthparts can cause infection. In this chapter the view will be adopted that insects inoculate viruses to plants solely by means of their mouthparts while feeding.

Although in Chapter 4 it was shown that a large number of viruses have now been shown to be transmitted by insects, detailed studies on the mechanisms of transmission and on the relationships between viruses and their vectors have been made on relatively few. However, sufficient has been done to show that viruses divide sharply into two distinct types on the basis of their relationships with their vector or vectors. Vectors of the first type may become infective by relatively short periods of feeding on infected plants, but they cannot infect other healthy plants immediately. Once they become infective they remain so for long periods, often for the remainder of their lives. Vectors of the second type of virus can infect plants immediately after a short period of feeding on a diseased plant and they cease to be infective in a short time, always less than 24 hours. These differences are undoubtedly determined by the viruses and not by the vectors, for the same insects sometimes transmit viruses of both types.

There is general agreement that insect-transmitted viruses are of two kinds, but individual workers have interpreted the facts differently and used different bases for separating the two types. At first it was believed that viruses of the first type had some complex biological relationship with their vectors, whereas the second type were transmitted purely mechanically as contaminants on the outside of the vectors' mouthparts (DOOLITTLE and WALKER 1928; HOGGAN 1933). This view is still widely held (*cf.* KUNKEL 1938; LEACH 1940), although there is considerable evidence that viruses of the second type are not merely transmitted mechanically and that the separation of viruses into those transmitted biologically and mechanically was premature. In the first edition of this book, the basis used for separation was a delay in development of infectivity in the vector. This delay was called an incubation period, and viruses were divided into those with and without incubation periods in their vectors. This is far from satisfactory, for the term incubation period implies a fixed time during which the virus develops or multiplies.

As great variations have been found for the time taken by individuals of one vector species to become infective with the same virus, the term incubation period is not suitable. Also, in the present state of our knowledge of the exact mechanism by which vectors transmit viruses, the implication that one type of virus develops or multiplies in its vector whereas the other does not, is decidedly premature. STOREY (1939) realised these uncertainties, and preferred a simple classification based on observed phenomena rather than on speculative interpretation. He again used the delay in developing infectivity as the basis of division but coupled it with retention of infectivity by the vectors. He defined the two types as (1) "Transmission negative on the first hosts, positive on succeeding hosts" and (2) "Transmission positive on first hosts, negative on succeeding hosts". Such a basis of classification, however, can only be used if the intervals of time spent by the vectors on each of a succession of healthy plants are suitably chosen for each virus. WATSON and ROBERTS (1940) and KASSANIS (1941), by using intervals of only a few minutes on each healthy test plant, have obtained infections on several successive plants with viruses that STOREY included in his second group. Similarly, if the time spent on the first host is long, some of the viruses in STOREY's first group will infect it. Thus, although the basis for separation does represent a very real difference between the behaviour of the two types of virus, without specified time intervals it loses much of its value.

WATSON and ROBERTS (1939) used the length of time that vectors remain infective as their basis for division. The first group, of which the vectors remain infective for long periods, they called "Persistent viruses"; the second, of which the vectors soon lose infectivity, they called "Non-persistent viruses". The ultimate result of any of these bases of separation is the same, but as the persistence or non-persistence of infectivity is perhaps the most striking difference and least likely to be obscured by the different techniques used by different workers, it seems the most suitable basis for division now available.

Viruses whose vectors remain infective for long periods:— The most important viruses whose vectors are known to remain infective for long periods are listed in Table 4. The further feature these viruses have in common is a delay in the development of infective ability. This delay between the times when the vectors acquire the virus and when they are able to transmit will be called a latent period, and in Table 4 the minimum latent periods described for the vectors of the viruses are given. It will be seen that the minimum varies from something less than 1 hour to 10 days. Experiments with single insects have often shown great variations between the latent periods of individuals of one species transmitting the same virus, and it is possible that some of the periods quoted are much above the minimum. Even so the differences are so large that they are probably significant.

Except for prolonged retention of infectivity and a latent period in their vectors, the viruses listed in Table 4 have little in common.

Their vectors are mainly leaf-hoppers, but aphids, whitefly and thrips also transmit some viruses of this type. Most of them either have not been transmitted by mechanical methods of inoculation or have been transmitted so only with difficulty, but tomato spotted wilt virus is an exception for it is readily transmitted by rubbing. Potato yellow dwarf virus can be transmitted between some hosts by rubbing and between others if the leaves are dusted with carborundum. Pea virus 1 can also be transmitted by rubbing if carborundum powder is incorporated in the inoculum, and sugar beet curly top virus has

Table 4:
Viruses with latent periods in their vectors.

Virus	Insect vector	Latent period	Reference
Abaca bunchy top	<i>Pentalonia nigronervosa</i> Coq.	24 hours	OCFEMIA and BUHAY (1934)
Aster yellows	<i>Cicadula sexnotata</i> Fall.	10 days	KUNKEL (1926)
Cereal mosaic	<i>Delphax striatella</i> Fall.	6 days	SUKHOV and SUKHOVA (1940)
Corn mosaic	<i>Perigrinus maidis</i> Ashm.	4 days	CARTER (1941)
Cotton leaf curl	<i>Bemisia gossypiperda</i> M. and L.	6 hours	KIRKPATRICK (1931)
Cranberry false-blossom	<i>Euscelis striatulus</i> Fall.	Undetermined	DOBROSKY (1931)
Maize streak	<i>Cicadulina mbila</i> Naude	6 hours	STOREY (1928)
Pea enation			
mosaic (Pea 1)	<i>Myzus persicae</i> Sulz.	12 hours	OSBORN (1938)
Peach yellows	<i>Macropsis trimaculata</i> Fitch	8 days	HARTZELL (1936)
Pineapple yellow spot	<i>Thrips tabaci</i> Lind.	Less than 10 days	LINFORD (1932)
Potato leaf roll	<i>Myzus persicae</i> Sulz.	24 hours	ELZE (1927)
Potato yellow dwarf	<i>Aceratagallia sanguinolenta</i> Prov.	9 days	BLACK (1938)
Rice stunt	<i>Nephotettix apicalis</i> Motsch	3 days	FUKUSHI (1935)
Sugar beet curly top	<i>Eutettix tenellus</i> Baker	4 hours	SEVERIN (1921)
Sugar beet leaf crinkle	<i>Piesma quadrata</i> Fieb.	Undetermined	KAUFMANN (1936)
Sugar beet yellows	<i>Myzus persicae</i> Sulz.	30 mins.	WATSON (1940)
Tomato spotted wilt	<i>Frankliniella insularis</i> Frankl.	5 days	BALD and SAMUEL (1931)

been transmitted a few times by specially designed injection methods believed to have introduced the virus directly into the phloem. Most of the viruses listed in Table 4 have not had their properties *in vitro* investigated, but there is great variability among those that have. Tomato spotted wilt and potato yellow dwarf viruses, for example, are inactivated in plant sap in a few hours at room temperature or by heating to 50° C, whereas sugar beet curly top virus remains infective for weeks and has a thermal inactivation point around 80° C.

It is probable that other viruses known to be transmitted by insects but not by mechanical inoculation, such as those causing rosette of lilies (OGILVIE 1928), strawberry yellow-edge (HARRIS 1933) and swollen shoot of cacao (POSNETTE 1941) are also of the type

whose vectors show prolonged retention of infectivity and a latent period, but they have not yet been examined in detail. Why mechanical transmission fails with so many viruses readily transmitted by insects is still obscure, but the vectors are largely phloem feeders and it is possible that for infection to occur these viruses must be injected directly into the phloem. STOREY (1938) states that the vector of maize streak virus can infect only if it feeds long enough to reach the phloem, and that if the insects feed on healthy plants through a membrane so that their stylets cannot reach the phloem they do not cause infection however long they feed. ROBERTS (1940) has shown that the chances of successful infection with sugar beet yellows virus are increased proportionally as the conditions of feeding increase the chances of the vector feeding on the phloem. Similar results have been obtained with sugar beet curly top virus. FIFE and FRAMPTON (1936) found that *Eutettix tenellus* fed on beet plants treated with carbon dioxide rarely succeeded in feeding on the phloem, whereas in normal plants they are always phloem feeders. On such treated plants infective vectors rarely caused infections.

According to BENNETT (1934) curly top virus occurs in much greater concentration in the phloem than in other tissues, and he suggests that other tissues may actually be toxic to it. With streak virus of maize there is no evidence for this, for the vector may pick up virus from tissues other than the phloem, although to infect healthy plants it must apparently place this virus in the phloem. The constant association of degenerative changes in the phloem of plants infected with some of these viruses, especially those causing sugar beet curly top and potato leaf roll, is a further indication of the close association of the viruses with the phloem. DYKSTRA and WHITAKER (1938) find that of the aphids known to transmit potato leaf roll virus, those that always feed on the phloem do so with greater regularity than those that may feed on other tissues (Fig. 14).

It has been suggested that viruses of this type are not transmitted mechanically because they are stable only when in the living plant or insect, and are inactivated immediately in expressed sap. The properties of tomato spotted wilt virus lend some support to this suggestion. This virus is readily transmitted by rubbing between certain host plants, although it has relationships with its vectors closely resembling those of viruses not transmitted mechanically, and it is inactivated in expressed sap more quickly than any other virus whose properties have been studied in detail. However, the suggestion is almost certainly wrong. BENNETT (1935) has been able to precipitate sugar beet curly top virus from expressed beet sap, and from extracts of macerated infective leaf-hoppers, and redissolve it in sugar solution so that the solutions are appetizing to the leaf-hoppers. By feeding *E. tenellus* on such extracts and then transferring them to healthy plants, he has obtained infections and has been able to study the properties of the virus *in vitro* in some detail. The virus will withstand drying, heating to 75° C, 28 days ageing at room temperature, 90% alcohol for 2 hours, pH changes between 3 and 9, and possesses considerable resistance to disinfectants. The failure of this virus to be transmitted mechanically obviously cannot

be attributed to its instability. Nor is maize streak virus quickly inactivated in expressed sap, for STOREY (1933) has made the interesting discovery that *Cicadulina mbila* can readily be infected by being injected with sap from diseased maize plants even though healthy plants cannot. Similarly, BLACK (1941) has transmitted aster yellows virus from infective to healthy vectors by inoculation methods. These results at first sight suggest that passage through the vector may change the infecting properties of the virus, in much the same way as the

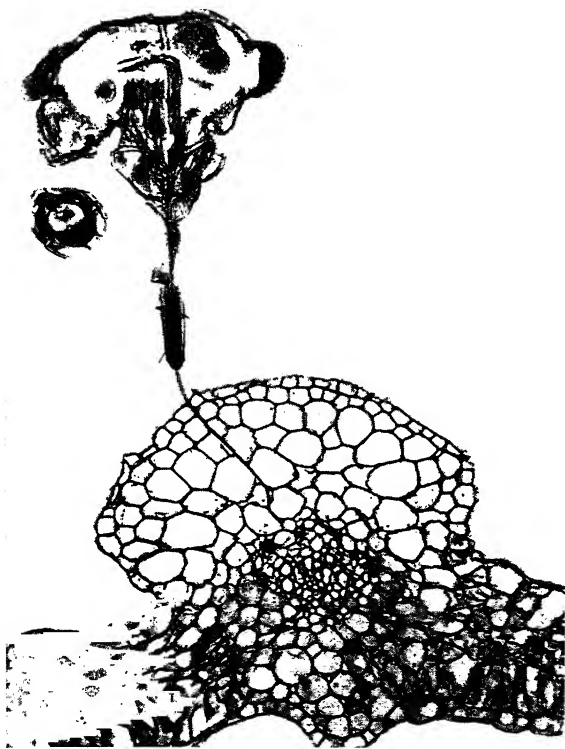


FIG. 14. — Section of a potato leaf showing the path of the stylet of *Myzus pseudosolani*. Note the path is intercellular through the cortex and that the aphid is feeding in the phloem tissue. $\times 80$. (DYKSTRA, T. P. and WHITAKER W. C., 1938, J. Agr. Res. 57, 319).

infecting powers of the spores of some rust fungi are altered by their hosts. The teleutospores of *Puccinia graminis* produced on wheat are unable to infect this plant, but readily infect the barberry. The barberry in turn produces the aecidiospores which will infect wheat but not barberry. It is possible then that the virus in the maize plant is in a form in which it can be transmitted by inoculation to *C. mbila* but not to plants, whereas in the insect it changes to a form readily inoculable to plants. On this theory the latent period could be explained as the length of time necessary for the developmental change in the virus to take place. There is no positive evidence for such a change. If it occurred it is to be expected that

plants would be susceptible to virus needle-inoculated from infective insects, whereas insects would not. In contrast to this expectation, STOREY has successfully inoculated healthy insects with the blood of infective vectors, but not plants. Similarly, *Eutettix tenellus* became infective when fed upon extracts of infected insects as readily as when fed upon diseased plant extracts.

The simplest, though by no means necessarily correct, explanation that can be offered for the ready transmission by insects of viruses not transmitted mechanically, is that they are unable to multiply in damaged cells. The feeding insects will probably produce less mechanical damage than any artificial method of producing entry points. But the fact that the insects have to place directly in the phloem viruses such as maize streak, which are known to be capable of infecting all tissue, suggests that they also cause too much damage to cells actually penetrated by the mouth-parts to allow the viruses to multiply. However, virus placed in the phloem could probably move out of such damaged areas much more rapidly than if placed in any other living tissues, and so enter uninjured cells of either phloem or other tissues in which it could then multiply. That some viruses of this type do move with great rapidity from their entry points is shown by the fact that curly top virus (SEVERIN 1924) and maize streak virus (STOREY 1928) have been detected at distances of 36 cm and 20 cm respectively from their entry points only one hour after infection. These rates of movement contrast remarkably with those found for viruses transmitted mechanically, for it is days after infection before these can be detected at such distances from their entry points. STOREY (1938) has also shown that cutting off the piece of leaf on which the vector of maize streak virus has fed has no effect in preventing infection. Such treatment would certainly prevent the infection of a plant rubbed with a virus that is transmitted mechanically. The two types of virus, therefore, seem to behave differently after entering infected plants. The mechanically transmitted viruses move only slowly from their entry points, whereas at least some of those transmitted only by insects move rapidly. It seems to be established that for the latter to cause infection they must be placed directly into the phloem. However, this cannot be taken as evidence that the phloem is the only susceptible tissue, for it may be that entry into the phloem is merely a necessary preliminary to entry into the most suitable uninjured tissues. A further possible explanation for the failure of mechanical transmission of these viruses has received little or no attention. Extracts of some plants contain substances that inhibit the transmission of even the most easily transmitted viruses. Preparations of tobacco mosaic virus, for example, can be rendered non-infective by the addition of sap from *Phytolacca decandra*. The virus is not destroyed by the sap, for by appropriate treatments it can be recovered fully infective. It is possible when sap is extracted from plants suffering from curly top, maize streak, aster yellows and other such diseases, that the viruses are brought into contact with substances that act as inhibitors, and that these are responsible for the failure of mechanical transmission. The vectors may either acquire the viruses free from

such inhibitors or have the power of separating the viruses from the inhibitors. If the second were true, the time taken for this separation could also account for the existence of a latent period. It must be emphasized that such a view is purely speculative, but it would seem worth investigating experimentally.

There is now conclusive evidence that for the vectors of sugar beet curly top virus and maize streak virus to become infective the viruses must enter the insects' blood. STOREY (1932, 1933) has shown that the ability of individual *Cicadulina mbila* to transmit maize streak virus is determined by the permeability of the insect's gut wall to the virus. He has found that the insect occurs naturally in two strains. These are indistinguishable from one another morphologically, and as they interbreed freely are almost certainly the same species. But the individuals of one strain are able to transmit maize streak virus, called active insects, whereas individuals of the other strain are unable to transmit (inactive). The ability to transmit is inherited as a simple dominant Mendelian factor linked with sex, the male being heterozygous for sex.

Individuals of both strains pick up the virus when feeding upon infected plants, but in the active insects the virus can be found in the contents of the gut and the blood, whereas in inactive insects it can be found only in the contents of the gut. When feeding naturally the inactive insects never transmit the virus, but by operating upon them STOREY has been able to turn them into vectors. If the gut wall of an inactive insect is punctured either before or soon after it has fed upon an infected plant, it behaves like an active insect. Virus can be detected in the blood of such insects and they are also able to transmit the virus. Similarly, if the virus is introduced directly into the blood of an inactive insect, by means of needles dipped either in infective maize sap or in the blood of infective insects, it is then able to transmit the virus to healthy plants. Both strains of the insect, therefore, can pick up the virus from infected plants, and both can transmit it to healthy ones. The sole difference between them seems to lie in the permeability of their gut wall, that of the active strain allowing the passage of the virus and that of the inactive preventing it.

The most probable sequence of events during transmission is this. The virus is sucked up through the mouthparts of the feeding insect and enters the gut. It passes through the gut wall and enters the blood, from which it passes into the salivary glands where it becomes mixed with the salivary secretions and is ejected back into plants when the vector again feeds.

The blood is most likely the main reservoir of virus in the infective insect, from which it probably passes only slowly into the salivary glands. STOREY has been unable to detect any virus in the salivary glands of *Cicadulina mbila*, and BENNETT and WALLACE (1938) found significantly less in the dissected salivary glands of infective *E. tenellus* than in other tissues. However, these tests were of necessity made on resting insects, and it is possible that the virus content of feeding insects, actively secreting saliva, might be higher. This possibility is strongly suggested by SMITH's (1941) results. He fed in-

fective *Eutettix tenellus* through membranes on drops of sugar solution for three hours, and then concentrated the solutions before allowing non-infective *E. tenellus* to feed on them. Of 40 insects fed on such solutions, 29 became infective. Although the salivary glands obviously can contain virus, especially in the feeding vectors, there is other indirect evidence that they have only a small content and are not the main reservoirs. If infective leaf-hoppers are fed on a series of healthy plants they usually fail to produce infection in every plant. Some individual leaf-hoppers infect more plants than others, and with all individuals the number infected increases with the length of time spent on the plants. It seems probable, therefore, that the salivary glands are easily exhausted of virus and that there is not a steady flow of virus from the blood to the glands, but that it takes place irregularly. If an insect is feeding on a plant while virus passes into its saliva then it is to be expected that the plant will be infected, but if it does not the plant will remain healthy. The probability of virus entering the saliva will increase with increasing time and will also be greater the higher the virus content of the blood. The variations in the ability of individual leaf-hoppers to cause infection in short feeding times, which have frequently been recorded, can be explained on this theory by variations in the virus contents of their blood or in some other factor affecting the rate of entry of virus into the salivary glands.

Apparently there is little wastage of virus by excretion in the faeces. STOREY (1932) found virus in the contents of the rectum of *Cicadulina mbila* if they had recently fed on infected plants, but none in the naturally voided faeces. SEVERIN (1931) also found no virus in the faeces of *Eutettix tenellus* carrying sugar beet curly top virus and BENNETT and WALLACE (1938) found only little.

There is one curious feature in the relationships of tomato spotted wilt virus and its vectors *Frankliniella insularis* and *Thrips tabaci* that has not been described elsewhere. Both the larvae and the adults can transmit the virus to healthy plants, but to become infective the thrips must feed on infected plants when they are larvae. The adults are unable to become infective (BALD and SAMUEL 1931). This is still unexplained, no differences in the feeding habits or anatomy having been found to account for it. But if STOREY's results with maize streak virus and *C. mbila* can be applied to spotted wilt virus, it may be explained on the difference in the permeability of the gut wall in the adult and larval thrips. The virus may be able to pass through the gut wall of the larva but not that of the adult. If this were so, then virus picked up by the feeding adult would either remain in the intestine or be excreted, the insect remaining non-infective. Whereas virus picked up by the feeding larva would penetrate the wall and enter the blood, and the insect would become infective. An adult from such a larva would already have the virus in its blood and so could continue to transmit, although no longer able to acquire further virus.

How viruses manage to pass through insects' gut walls is unknown, for *in vitro* they are colloidal and do not diffuse through semi-permeable membranes. No signs of any injury or lesion have been found in

the gut walls of infective insects. The absence of a peritrophic membrane in vector species of insects may render penetration easier than in other species, but the possibility that the virus plays an active rather than a passive part cannot be ruled out. It is possible that the ability to pass through the gut wall determines the specific relationships between viruses and their vectors. For example, potato leaf roll virus may be able to penetrate the gut wall of *Myzus persicae* but not of *Eutettix tenellus*, whereas curly top virus may be able to penetrate the wall of the latter but not of the former. It has been definitely established that non-vector species of insects pick up viruses while feeding upon infected plants. STOREY (1933) has found maize streak virus in the intestine, but not in the blood, of *Peregrinus maidis*, and BENNETT and WALLACE (1938) have found curly top virus in a number of species of aphids, thrips and leaf-hoppers after feeding on infected plants. From some of these non-vectors appreciable quantities of virus were recovered as long as a fortnight after they had access to a source of virus. The inability to transmit, therefore, cannot be explained on the assumption that non-vectors destroy these viruses. However, factors other than the permeability of the gut wall to the particular virus are probably involved, for STOREY was unable to convert *P. maidis* into a vector by puncturing its gut wall as he could the inactive strain of *Cicadulina mbila*; nor did *P. maidis* transmit when maize streak virus was injected directly into its blood.

Multiplication of viruses in their vectors:— Various reasons have been advanced to account for the latent period of the viruses in their vectors. One of these, that it is the time taken for the virus to undergo a developmental change in the insect, has already been discussed. Another is that it represents the time required for the viruses to enter the blood and reach the saliva. In the writer's opinion this is most probably correct. Unless different viruses take remarkably different times to pass through the gut wall of their vector, however, it is difficult to explain the large differences in the incubation periods of different viruses. A third, and probably the most widely accepted, explanation is that the vector while feeding on infected plants acquires insufficient virus for it to be able to transmit, and the latent period is regarded as the time required for this amount to multiply in the insect sufficiently for it to become infective.

The fact that once the vectors have become infective they remain so for long periods at first sight also suggests that the virus multiplies in them. However, infective vectors have often been found to lose their ability to infect plants, and work by FREITAG (1936), BENNETT (1938) and STOREY (1938) suggests that this fact can be interpreted differently. These workers find that the length of time leaf-hoppers remain infective depends upon the length of time they have fed upon the source of the virus. They may become infective after a few minutes' feeding but if so they do not remain infective for long. By contrast, if they have fed for hours or days they remain infective for long periods, often for the remainder of their lives. Working with sugar beet yellows virus WATSON (1940) also found

that both the efficiency of the vectors and the length of time for which they remained infective increase with increased feeding time on the infected plant. The chances of transmission are also increased by prolonging the feeding on the healthy test plants. She got occasional infections with insects given a total feeding time of 30 minutes on the diseased and healthy plants, but the numbers increased greatly when the times on either were increased. WATSON challenges the whole conception of a fixed latent period, and suggests in its place a time during which the chances of successful transmission increase until a maximum is reached at which all insects capable of transmitting will have done so. Whether insects can infect only one or many plants will depend on how long they have fed on the source of infection, but when they cause infections will depend solely on the chance ejection of virus into the healthy plants. The results with curly top virus also fit with this idea. Most workers have found a latent period of at least 6 hours for the virus, but SEVERIN (1931) got some transmissions with vectors fed for only 10 minutes successively on infected and healthy plants. He concluded that these were due to some anomalous method of transmission, possibly mechanical transfer on the vectors' mouthparts. WATSON (1940) has pointed out that if two methods of transfer were involved there should also be two optima in the curve number of transmissions plotted against time. Instead, with both curly top and yellows viruses, the curves are smooth, rising steeply at first and then flattening out towards a maximum.

Although the length of time that the vectors feed on the infected plant may determine for how long they remain infective, it has little or no effect on the duration of the latent period (FREITAG 1936; STOREY 1939), which seems to start from the time the vectors leave the infected plant rather than from the start of feeding on it. If the latent period were merely the time taken for the virus to reach an infective concentration, there seems no reason why the whole of the period should not be passed on the diseased plants and why adult insects raised on diseased plants should not infect healthy plants immediately. There have been no detailed experiments on the interactions between the lengths of infection feeding and duration of latent periods with viruses such as aster yellows whose vectors have prolonged latent periods, but in published work there is no indication that vectors can ever infect healthy plants immediately after leaving infected ones.

These facts are all difficult to reconcile with the view that viruses multiply in their vectors and that the latent period is the time taken for the viruses to reach an infective concentration. A plant inoculated with a minimum infecting dose of virus takes longer to show systemic symptoms than one inoculated with a massive dose, but ultimately the two plants come to have the same appearances and virus-contents. Therefore, if the viruses multiplied in their vectors as they do in susceptible plants, it might be expected that the latent period would be lengthened by short feeding periods on infected plants. But there is no obvious reason why an insect which has acquired sufficient virus to become infective should not ultimately

have the same virus-content, and remain infective for as long, as one receiving a larger initial dose of virus.

A more reasonable interpretation of the observed phenomena is that vectors contain no more virus than they take up while feeding on infected plants, and that this amount is either stored in some tissue or circulates in the blood, to be dissipated only slowly and irregularly through the salivary glands. The reasons for the latent period are obscure, but on this view it could be regarded as the time taken for the virus to reach the saliva in an infective form. If this time is independent of the length of the infection feeding, it is possible that the insects imbibe some inhibitor from the diseased plant whose influence has to be removed before it can cause infection. There may be some loss of virus by destruction in the vector or by diffusion into tissues other than the salivary glands, but the loss during feeding will probably be greatest. If passage into the saliva is gradual and discontinuous, the prolonged infective state of insects given long infection feeding is readily explained, for there is good experimental evidence that insects can acquire from infected plants sufficient virus to infect a large number of healthy plants. BENNETT (1935) has shown that *Eutettix tenellus* fed artificially on phloem exudate diluted to 1/10,000, picked up sufficient virus to infect healthy sugar beet with curly top virus. Had the insects been feeding on diseased plants for the same length of time they would have been imbibing undiluted phloem contents. It is not unreasonable to assume, therefore, that they would have picked up something like 10,000 times the minimum amount of virus necessary to cause infection. As there appears to be little loss of virus in the faeces from feeding insects, it seems that most of the virus imbibed must enter the blood; therefore, providing only that the loss of virus from the blood to the saliva is slow, in relatively short feeding periods it will be possible for the vectors to accumulate more than sufficient virus for them to continue infecting plants for the remainder of their lives.

The work of BENNETT and WALLACE (1938) provides further evidence that insects can acquire much more virus from infected plants than they need to reach their maximum infectivity. The ability of leaf-hoppers to infect large numbers of plants with curly top virus increases rapidly with the length of time they are allowed to feed on the source of the virus, and reaches a maximum in about two days. But if they are allowed to continue feeding on the source of the virus, the virus-content of the leaf-hoppers (measured by the ability of other leaf-hoppers to become infective when fed upon extracts of the infective hoppers) goes on increasing until they have fed for a fortnight, although their ability to infect healthy plants is not increased. As this increase occurs only when the infective insects are continuously fed upon diseased plants, it is obviously the result of more virus taken in by the mouth and not to any multiplication in the insects.

Most workers have found that the progeny of infective vectors are free from virus provided that they are removed from diseased plants before they have fed, or provided that they are born on healthy plants. According to FUKUSHI (1934; 1935) the virus causing dwarf

disease (stunt) of rice is an exception and is frequently transmitted through the eggs of infective *Nephotettix apicalis*. Transmission to the progeny is determined solely by the female, crosses between infective males and non-infective females always giving virus-free offspring. Not all the progeny of infective females are infective, but sometimes the virus is handed down through several generations of leaf-hoppers without further access to a source of virus. FUKUSHI regards this as strong presumptive evidence that this virus multiplies in its vector. However, as he also finds that infective leaf-hoppers often not only fail to transmit to their progeny but themselves cease to be infective, even in favourable conditions, it seems improbable that the virus does multiply. Unfortunately, no attempt has been made to correlate the length of time *N. apicalis* remains infective, or its ability to transmit the virus to progeny, with the length of time it has fed on the diseased plants, but the feeding times mentioned are days or weeks. It may be that those fed for long periods, so acquiring a high charge of virus; are those that transmit for their whole lives and transmit to their progeny. Similarly, those that feed for short periods may be those that lose their infectivity. It has been shown above that *Eutettix tenellus* in long periods of feeding can acquire many times the amount of curly top virus necessary to make it infective for the whole of its life. If it is assumed that *Nephotettix apicalis* can also do this with the rice-stunt virus, then, as this virus apparently can enter the eggs of this species, there seems no obvious reason why an insect fed long enough on a diseased plant should not acquire sufficient virus to render its progeny infective. Furthermore, if the virus does multiply in the insect there seems no reason why transmission should be limited to any number of generations. Indeed, an insect born infected would be expected to have a greater chance of producing infective progeny than one which only becomes infective in later life by feeding on infected plants. This effect is clearly shown in bean plants suffering from common mosaic. Here the virus is seed transmitted and does multiply in the bean plant, and the amount of seed transmission is greater in plants raised from infected seeds than in those that become infected during the growing period.

Some infective nymphs of *N. apicalis* can infect healthy plants immediately they emerge from the eggs, whereas others do so only after a variable waiting period. This at first sight again suggests multiplication of the virus in the insect, for the newly-hatched nymph may contain too little virus to cause infection, and the waiting period may be the time required for this virus to multiply. But there are other equally probable interpretations. Newly-hatched nymphs may contain different initial charges of virus or the virus they contain may be distributed differently. A nymph born with a high virus content or with virus already in the salivary glands might be expected to transmit immediately, whereas it might be a considerable time before the virus in an insect with a low virus-content entered the salivary gland and the insect could transmit.

KUNKEL (1937, 1938) has found that *Cicadula sexnotata* carrying aster yellows virus are unable to transmit if exposed to high tempera-

tures. Leaf-hoppers exposed to a temperature of 32°C for a day lost the ability to infect healthy plants, but on lowering the temperature to 24°C they quickly regained the ability without again feeding on a source of the virus. Infective leaf-hoppers kept at the high temperature for a week regained the ability to transmit when subsequently kept at the lower temperature, but they regained it much more slowly than those heated for one day. Leaf-hoppers heated for one day regained the ability to transmit in a few hours at the lower temperature, those heated for a week required two days or longer, whereas those heated for more than 12 days never regained the ability to transmit.

KUNKEL regards these facts as strong evidence that the virus multiplies in the insect vector. He interprets his results as indicating that long heat treatments cause inactivation of all the virus in the infective insect and that short treatments cause inactivation of a part only. The time required for treated insects to regain the ability to transmit is regarded as a latent period during which the non-inactivated virus multiplies sufficiently to render the insects infective. This interpretation seems to the writer to necessitate a number of assumptions for which there is no positive evidence. Firstly, as the ability of the vector to infect plants is destroyed by a short period of heating and is regained in a few hours, destruction of a small amount of virus must be assumed to render the vector non-infective. Thus it appears that the fully infective insect can be carrying only the minimum charge of virus necessary to infect a plant, a conclusion widely differing from the results of BENNETT with *Eutettix tenellus* which show that this leaf-hopper can carry many times the charge of virus necessary for its maximum infectivity. Secondly, it seems necessary to assume that the virus multiplies much more readily in the insect than in susceptible plants. For KUNKEL's results show that insects treated for one day cease to be infective, and on his interpretation contain too little virus to cause infection in a plant. Yet insects treated for a week still contain sufficient virus to re-infect and multiply in themselves.

KUNKEL found that the leaf-hoppers lived and bred for long periods at the high temperature without apparently being harmed, and because of this he states the effect of heat must be on the virus. Even if this is assumed, however, and the loss of infectivity is regarded solely as a result of virus inactivation, there would seem to be no reason for assuming that the recovery of infectivity is a result of virus multiplication in the vectors. The body of an insect is such a complex system and so little is known about it that there are many other equally probable explanations. Virus in or near the salivary gland may be inactivated by heat more rapidly than virus in the blood. Then the loss of infectivity after short heat exposure could be explained by the inactivation of the virus in the gland; and the waiting period as the time required for virus again to enter the saliva. If continuous heat inactivates the virus in the blood, then with decreasing virus concentration the probability of virus entering the saliva from the blood will be reduced, and the length of time required for the virus to enter the saliva, and for the insect to become infective,

will be increased. But heating may also have effects other than inactivating the virus. The insect's blood or tissues may be affected so that the virus is more firmly absorbed, or precipitated. If this were so then a sufficient length of time would be necessary for the insect to return to normal before the virus was liberated and free to enter the saliva. Or again heating may make the salivary gland less permeable to the virus. Indeed, there are so many possible alternative explanations of these results that it would seem extremely premature to regard them as evidence for the multiplication of the virus in the vector.

The view that aster yellows virus multiplies in its vector has been supported by BLACK (1941), who claims to have demonstrated that the virus multiplied at least a hundred-fold between the second and twelfth days of the latent period. *Cicadula sexnotata* were fed on diseased asters for one or two days and then removed to healthy plants. At intervals, batches of insects were macerated and their extracts diluted and inoculated into non-viruliferous individuals, which were later tested for their ability to produce yellows in asters. Insects macerated before the fourth day gave no infection, whereas those macerated on the twelfth day gave infections when diluted 1/1000.

At first sight this looks convincing evidence of multiplication, but closer examination of BLACK's tables suggests other interpretations and clearly shows that even if multiplication occurs it is only to a very limited extent. Firstly, it is noticeable that the number of successful inoculations is usually greater if the extracts of macerated insects are diluted 1/1000 than if diluted 1/100 or 1/10. Thus it seems clear that the difference between a successful and unsuccessful inoculation cannot be directly attributed to differences in virus content, for increased dilution can hardly be expected to increase the virus content. These results strongly suggest that the juice of macerated insects contains some inhibitor of infectivity, which becomes less effective as the latent period progresses and whose inhibitory action is reduced by dilution. Inhibitors with the second quality are well known and have been shown by BLACK (1939) to occur in extracts of insects, for he found that the infectivity of mixtures of tobacco mosaic virus and clover leaf-hopper juice can be increased by dilution. Secondly, the infectivity of vectors fed for long periods on infected asters, used by BLACK as controls for his inoculation technique, was strikingly higher than that of his test insects which had fed for only one or two days on infected plants. Thus with aster yellows virus, as with curly top virus, it seems that the virus content of the vector is largely determined by the feeding time on the diseased plant; if there is any multiplication during the latent period, it is less effective in increasing the virus content than continued feeding on the diseased plant.

With viruses such as maize streak and aster yellows, which can be mechanically transmitted to their vectors, experiments could be made to settle the problem of virus multiplication in the vector. Vectors could be inoculated with various dilutions of virus-extracts, and after a suitable lapse of time they could be macerated and their

extracts inoculated at various dilutions to non-viruliferous vectors. If the vectors inoculated with the minimum concentration of virus ultimately came to have the same virus content as those inoculated with concentrated extracts, there would be good evidence of multiplication. Secondly, insects could be inoculated with dilute extracts, then macerated after a suitable time and their juice again diluted and inoculated to fresh insects. If this process succeeded in producing a long series of infective insects, as it undoubtedly does of plants, the evidence for multiplication in the vector would be unquestionable. Until such proof is forthcoming, the balance of evidence is against multiplication on any considerable scale, although the possibility of limited multiplication with some viruses cannot definitely be excluded. All the presumptive evidence offered in support of such a view, however, can be interpreted differently.

Most workers have failed to find any significant differences between infective and non-infective individuals of vector species. Infective individuals live as long and breed as freely as non-infective. No morphological changes or disease symptoms have been found. This, of course, cannot be taken as evidence that the viruses do not multiply, for viruses multiply freely in many plants (carriers) in which they cause no symptoms. HARTZELL (1936, 1937) claims to have found intracellular inclusions in *Macropsis trimaculata* carrying the virus causing peach yellows. These inclusion bodies are similar to those described by HARTZELL in diseased trees, but they differ so widely from those described in other virus diseases that until more evidence has been obtained about them it would be dangerous to accept them as true virus inclusions.

Viruses whose vectors soon lose infectivity:—The vectors of this second type of virus behave quite differently from those of the first type and can cause infection immediately after a short feeding time on the infected plant. Indeed, the less the delay in transferring insects from the infected to the healthy plants, the greater is the probability that they will cause infection. By making use of the different vector-relationships of the two types of virus, it is often possible, using only one vector species, to separate pure cultures of viruses from plants infected with mixtures. *Myzus persicae* fed for an hour or so on potato plants infected with both leaf roll virus and potato virus "Y" will probably pick up both viruses. If the aphids are immediately transferred to a series of healthy plants, on the first of which it stays for not more than twelve hours, the first plants will become infected with potato virus "Y" only. After a day or more the aphids become able to transmit leaf roll but have now ceased to transmit virus "Y", and subsequent plants on which they feed are infected with leaf roll virus only.

Not all of the viruses whose vectors remain infective for short periods only have had their properties in expressed sap examined, but those that have form a fairly uniform group (WATSON and ROBERTS 1939). They are inactivated by ten minutes' heating at temperatures about 55° C or by a few days ageing at room temperature, and they are easily destroyed by acid and alcohol. All these viruses have

aphids for vectors and they are also readily transmitted between plants by rubbing or needle-inoculation. This has led many workers to suggest that the vectors act in a purely mechanical manner, merely picking up the virus as a contaminant on their mouthparts in the same way as a needle might when inserted into the plant. The fact that two minutes' feeding on an infected plant followed by five minutes on a healthy plant often results in infection, shows that the mechanism of transmission must differ profoundly from that of viruses with latent periods. Also, as the vectors soon cease to be infective there is obviously no need here to discuss the possibility of these viruses multiplying in their vectors. Nevertheless, there is now a good deal of evidence that vectors of these viruses do not merely behave as needles.

If the vectors were acting merely mechanically it is difficult to see why all insects feeding similarly should not be equally efficient vectors, except that those with larger mouthparts might be expected to carry a little more virus. However, even with viruses which are transmitted by a number of species of aphids, one species is often a more efficient vector than the others, and this efficiency cannot be correlated with size. SEVERIN and FREITAG (1938) transmitted western celery mosaic with eleven different species of aphids, but the percentage infection obtained with individual species varied between 14 and 84. Sometimes two aphids are able to transmit the same two viruses, but one aphid will transmit the first virus more efficiently than it will the second, whereas the other will transmit the second more efficiently than the first. WATSON (1938*b*) finds that *Myzus persicae* is a more efficient vector of Hyoscyamus virus 3 than is *Myzus circumflexus*. Neither insect transmits cucumber virus 1 as readily as Hyoscyamus virus 3, but *Myzus circumflexus* is at least as efficient, and probably more so, than *Myzus persicae* in transmitting cucumber virus 1. It appears, therefore, that there is some kind of relationship between these viruses and their vectors.

Also, if transmission by insects were merely mechanical, it might be expected that viruses most readily transmitted by artificial inoculation would be most readily transmitted by insects. And, if the virus were merely acquired as a contaminant on the mouthparts, the viruses occurring in infected plants in the greatest concentration might be expected to be most easily acquired and transmitted. But it is not so. Tobacco mosaic virus and potato virus "X" are more easily transmitted by rubbing or by needle inoculation, and occur in greater concentration in extracts of infected plants, than any of the viruses under discussion. Neither of these normally appears to be insect transmitted. HOGGAN (1931) has shown that *Myzus persicae* fed on tobacco infected with both tobacco mosaic virus and cucumber virus 1 transmit only cucumber virus 1, and SMITH (1931) could always get pure cultures of potato virus "Y" by feeding *Myzus persicae* on plants infected with both this and potato virus "X". By contrast, if inoculations were made with needles by first pricking plants infected with these pairs of viruses and then pricking healthy ones, the viruses most likely to be transmitted are certainly tobacco mosaic and potato "X".

These two viruses have been studied in detail by many different

workers, but there is only one serious claim for transmission by insects. HOGGAN (1931) found that a number of aphid species all failed to transmit tobacco mosaic virus from infected tobacco plants. However, when fed on infected tomato plants infections were regularly obtained with *Myzus pseudosolani* and *Macrosiphum gei*, but not with *Myzus persicae*. *Myzus pseudosolani* gave consistently more infections than the other aphids. These rather curious results have not been confirmed, but if the transmissions are accepted as true insect transmissions and not accidental contaminations, again they do not suggest that the virus is carried mechanically on the mouthparts. For the varying ability of the different aphids to transmit, together with the fact that the insects do not become infective when fed on tobacco, although the virus content of sap from tobacco infected with this virus is greater than tomato, suggest some relationship between the virus and vectors. In these experiments very large numbers of aphids were transferred from infected to the healthy test plants. In spite of this, all the test plants rarely became infected as they did in similar experiments with cucumber virus 1. A possible explanation for these results is that most of the individuals of the aphid species are not vectors of tobacco mosaic virus, or in STOREY's terminology are "inactive", whereas occasional individuals may be active. Then increasing the number of aphids transferred would increase the probability of including an active individual and increase the probability of getting infection. The failure of workers other than HOGGAN to transmit tobacco mosaic virus with aphids may be due to the smaller number of insects used or to their cultures containing no active individuals.

If insects transmit viruses merely by adsorbing infective plant sap on to their mouthparts, it is difficult to understand why tobacco mosaic virus should not be transmitted by any insect. On the other hand, if the viruses must be sucked up and then ejected possible explanations can be offered for the fact that insects do not normally transmit this most infectious virus. It is possible that the virus is quickly inactivated in the intestinal tract of aphids. It is unlikely to be destroyed, for *in vitro* tobacco mosaic virus withstands treatments that rapidly destroy most insect-transmitted viruses, but it is possible that the secretions of aphids contain substances that specifically inhibit its infectivity. Another possible explanation is that vectors of other viruses are unable to ingest tobacco mosaic virus. BENNETT and WALLACE (1938) have shown that leaf-hoppers and aphids, which do not transmit curly-top virus, do contain this virus after feeding on diseased beet, and SMITH (1941) has found that tobacco mosaic virus passes through the alimentary canal of caterpillars, but it still remains to be shown that sucking insects fed on plants with tobacco mosaic contain any virus.

Considerable evidence for the view that viruses whose vectors soon lose infectivity have more than mechanical relationship with their vectors has been got by WATSON (1936, 1938*a* and *b*) and WATSON and ROBERTS (1939, 1940). This work was chiefly done with *Hyo-scymus* virus 3 and *Myzus persicae*, but similar results were got with potato virus "Y", cucumber virus 1 and tobacco etch virus,

with *Myzus persicae* and other aphids. After feeding on infected plants, aphids were transferred singly to the healthy test plants, only one aphid being placed on each plant, and the efficiency of the vectors was measured by the number of healthy plants out of a hundred infected by single aphids. Working with single aphids in this way it was possible to control the length of time they fed on both the source of the virus and on the healthy test plants. The results obtained are the most unexpected and contrast remarkably with those obtained with viruses having latent periods.

The efficiency of the vectors is much increased if they are prevented from feeding for some time before they are fed on the source of the virus. As short a fasting period as 15 minutes has a pronounced effect on the percentage of transmissions. Little further increase in efficiency occurs after one hour's fasting, but this treatment may result in from 7 to 9 times the number of infections that would be produced by unstarved aphids. This remarkable effect of fasting is obtained only if the insects are fed on the source of the virus for short periods before being transferred to healthy test plants. The optimum conditions for transmission are to use insects that have previously fasted for at least one hour, feed them on the diseased plant for about 2 minutes and then transfer them immediately to the healthy test plants. Increasing the length of time the aphids feed on the infected plant greatly reduces the number of infections obtained, and if they are allowed to feed for as long as an hour, insects starved before use give no more infections than those unstarved. The reduction in the efficiency of the vectors, therefore, is produced by continuous feeding and it is immaterial whether the aphids feed continuously on infected or on healthy plants. The optimum conditions for transfer are such that can occur seldom in nature with wingless insects, and even with winged insects it must be relatively rare for an insect having ceased feeding for an hour to alight on the infected plant, feed for only a few minutes and then fly off to a further plant. The fact that conditions in nature normally reduce the efficiency of aphids as vectors may explain the relatively slow spread of this type of virus even when the vector is present in large numbers.

The results with insect transmitted viruses such as Hyoscyamus virus 3 are directly opposite to those with curly top of sugar beet and maize streak. The efficiency of the vectors of the first decreases rapidly with the length of feeding time on the source of the virus, whereas the longer the vectors of the second feed the more efficient vectors they become. It seems improbable, therefore, that the ability of *Myzus persicae* to transmit Hyoscyamus virus 3 can be dependent solely on the concentration of virus. For it is apparent that in long feeding periods the aphids will have chance to acquire more virus than in short periods, but after long periods they are less efficient vectors.

Infective aphids can infect several healthy plants in succession without again having access to a source of the virus, provided that they feed for only short periods on each plant (WATSON and ROBERTS 1940; KASSANIS 1941). The rate at which they lose their ability

to transmit is also determined by their feeding. Aphids fed for a few minutes on the source of the virus and starved before and after this feeding period may remain infective for 12 hours, whereas those allowed to feed continuously either before or after feeding on the source of the virus cease to be infective within an hour.

It seems impossible to reconcile these results of WATSON with the view that the viruses are merely carried as contaminants on the outside of the insect mouthparts. For there is no apparent reason why the mouthparts of starved aphids should absorb more virus than those of unstarved. Neither is there any obvious reason why the stylets of starved aphids should absorb from a plant more virus in 2 minutes than in 1 hour, nor why virus should disappear from the stylets of unstarved insects in an hour and take 12 hours to disappear from those of starved insects. To explain these results it seems necessary to assume that the virus transmitted by the aphids is taken in by the mouthparts and then injected back into plants. Then, as WATSON has shown, the results can be explained by postulating something in the aphid which destroys the infectivity of the virus, the something being present in relatively large quantities in aphids which have fed continuously for some time but absent from, or present in smaller quantities in, aphids which have been starved. The something need not necessarily destroy the viruses, for it might behave like trypsin and certain proteins, merely reducing infectivity by acting on the plant or by forming a non-infective complex with the viruses.

Such inhibitors do occur in the bodies of insects, both vector and non-vector species. WATSON (HAMILTON 1934) showed that crushed juice from *Myzus persicae* rendered sap from plants infected with *Hyoscyamus virus 3* non-infectious and SMITH (1941) found that extracts from caterpillars neutralised the infectivity of tobacco mosaic and tobacco necrosis viruses. BLACK (1939) has also shown that juice from macerated leaf-hoppers and aphids inhibits tobacco mosaic virus and that several other viruses are also inhibited by extracts from the clover leaf-hopper. There is no evidence that in normally feeding vectors the viruses ever come into contact with the inhibitors found in macerated insects, but their existence lends support to WATSON's suggestion.

There is further evidence that continuous feeding reduces efficiency as a vector, although it must increase the amount of virus acquired by the vector. WATSON has shown that the longer the aphids feed continuously on diseased plants the less efficient they become in transmitting. On the other hand, if they are given two or more short infection feedings and are starved before the first and between the subsequent feedings, their efficiency is increased. From this it appears that the aphids have acquired more than their usual dose of virus by having the short interrupted feedings, but have avoided the deleterious effects produced by continuous feeding. Aphids which have once been infected can again transmit if given a second feeding on infected plants (KASSANTIS 1941), so that the loss of infectivity is not the result of any permanent change in the vector.

The plant-tissue relationships of these viruses during transmission

also differ from those of viruses having incubation periods in their vectors. The aphids can become infective by feeding on a diseased plant, and infect a healthy plant, in feeding times too short for their stylets to reach the phloem. At first sight this suggests that aphids feeding for short times may have their stylets in tissues more favourable for picking up the viruses than those feeding for long periods, and so explain the fall in infectivity accompanying long infection feedings. But if this were so the fall would be expected with all insects and not only with the starved ones. Also, the effect of continuous feeding on the efficiency of the vectors would be determined solely by the time they fed on the infected plant, whereas it is actually determined equally by continuous feeding on healthy plants before access to infected plants. Working with various strains of *Hyoscyamus virus 3*, cucumber virus 1 and potato virus "Y", WATSON and ROBERTS (1939) found that the efficiency of vectors was correlated with the virus content of the sap in plants on which the vectors fed. This apparently is not a general correlation, however, for KASSANIS (1941) found that mild etch and severe etch viruses were transmitted with equal readiness, although sap from plants infected with the latter contains many times as much virus as the former.

The mechanism of transmission of this type of virus is still quite obscure. The evidence suggests that the viruses must be sucked up through the vector's mouthparts and not just carried on the outside, but how they get into a position in which they can be ejected back into a plant is unknown. The short time elapsing between feeding on an infected plant and infecting a healthy plant would seem to preclude the penetration of the gut wall, circulation in the blood and entry into the salivary glands as was suggested for viruses with significant incubation periods in their vectors.

Effect of the number of insects in transmitting:—Many workers have found that they get transmission of viruses fairly readily when large numbers of insects are transferred from diseased to healthy plants, but get few or none when single insects are used. This has often led to the suggestion that insects can inject sub-minimal infection doses of viruses into plants, the effects being cumulative so that a plant receiving sufficient of these doses will become infected. And it is implied that the infection produced by a group is contributed to by all the members, none of which could have produced it alone.

There is now strong evidence against this view. WATSON (1936) finds that with *Hyoscyamus virus 3* and *Myzus persicae* the percentage of plants infected increases regularly with the number of aphids placed on each healthy plant. In experiments in which 1, 5, 10 and 20 aphids were used per plant the numbers of plants infected agreed well with the numbers expected on the basis that each infection was local and independent. There was no large increase when large numbers of aphids were placed on each plant as would occur if the effects of individuals were cumulative. By contrast, the number of infections obtained when large numbers of aphids were placed on each plant was rather smaller than would be expected

from the number produced by single aphids, although not significantly so.

With maize streak virus and *Cicadulina mbila* STOREY (1938) also finds that the probability of infection by a group of leaf-hoppers is the probability that one or other of the members of the group would alone cause infection. From experiments in which individual infective leaf-hoppers were allowed to feed several times, he further concludes that the probability of a single leaf-hopper causing infection in a number of distinct feeding periods is the probability that it would cause infection in one or other of those feeding periods. These results seem to support the suggestion made earlier in the chapter that the passage of the virus into the saliva takes place irregularly. For they imply that the insect injects virus into the plant in distinct doses, the effect of each being independent of any other dose that may be injected by the same or another leaf-hopper. If the virus were continually present in the saliva and being injected into the plant at a steady rate, whether or not a leaf-hopper caused infection in a single feeding period would depend solely on the length of the feeding period. All infective leaf-hoppers cause infection if allowed to feed on healthy plants for extended periods. If single leaf-hoppers are given a number of short infection feedings of different lengths, however, infection does not necessarily occur in the longer periods. Infection often occurs in some feeding periods of about ten minutes and not in others of from four to five times as long. From this it seems that the ability of insects with their stylets in positions favouring infection to cause infection does not depend merely on the amount of saliva ejected, but on the chance of some of the saliva containing virus.

It is probable that the mass-action effect suggested by earlier workers is not a real effect, but was obtained because experimental conditions were unfavourable for large numbers of transmissions. If conditions were such that only ten per cent of the insects could transmit then most of the healthy plants colonised with a hundred insects would become infected, but only ten per cent of the plants colonised with single insects would.

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Chapter VI

VIRUS STRAINS, MUTATION, AND ACQUIRED IMMUNITY

Long before the existence of viruses was recognised, it was noticed that a person who had once suffered from smallpox rarely contracted the disease a second time, even though he was frequently exposed to infection. This phenomenon is now known to be fairly general, though not universal, with the virus diseases of man and animals, for recovery from many confers a lasting immunity against subsequent infection. Because of this, work on animal viruses has tended to be dominated by immunological studies, mainly devoted to the discovery of methods whereby the immunity could be conferred without exposure to the grave risks accompanying infection with virulent pathogens. Such work soon led to the discovery that many of the animal viruses were not fixed entities, but that they could be made to vary and exist in strains of different virulence. In the eighteenth century the principle of vaccination against smallpox was introduced by JENNER. He showed that cow-pox produced only a mild reaction in man, but after infection an immunity was produced against the dreaded smallpox. About 56 years ago PASTEUR did the first experimental work on alteration of viruses, and he found that rabies could be greatly modified by repeated passage through the brains of rabbits. In this way from the naturally occurring virulent form he produced the attenuated or "fixed" form, which is still used as a vaccine. Since then numerous variants of other animal viruses have been discovered. Some of these have been found occurring in nature whereas others have been produced experimentally, but there are now few for which no variants have been described.

Virus diseases of plants differ strikingly from those of animals, for systemically infected plants usually contain active virus for as long as they live. A few instances have been recorded in which plants have recovered, and become virus-free, but where these have been tested they have always been found to be susceptible to subsequent infection with the same virus. There is no reason to regard this as evidence that the viruses attacking plants differ fundamentally from those attacking animals, for the bacterial diseases of plants and animals differ in essentially the same manner. The difference lies in the defence mechanisms possessed by animals but not by plants. In response to infection, animals produce specific antibodies capable of uniting with and neutralising the effects of pathogens. The antibodies produced by an animal against one strain of a pathogen can often neutralise the effects of other strains, although they have no effect on unrelated pathogens. And it is to the presence of these antibodies in their blood stream that animals which have been vaccinated, or which have recovered from a disease, owe their sterile

type of immunity to subsequent infection. There is no evidence that plants do produce such antibodies. Hence the viruses can usually persist and multiply indefinitely in infected plants. The few plants that for unknown reasons occasionally recover contain nothing capable of combining with the viruses, and so are as susceptible to infection as plants that have never been infected. Because of this difference, there has been in the past little hope of controlling plant diseases by the vaccination methods so successfully applied to many animal diseases. Consequently, there has been little incentive for the discovery of avirulent strains of plant viruses as there has been with animal viruses.

In recent years, however, the outlook has rather changed, and this type of work has been actively pursued. There is still no evidence that plants can produce antibodies to viruses and acquire the sterile type of immunity. But it has now been shown that plants can be protected against virulent viruses to which they are normally quite susceptible. This has resulted from the discovery that plant viruses, like animal viruses, are not fixed entities, but can vary and exist in strains differing widely in virulence. For it has been shown with a number of viruses that plants fully infected with avirulent strains may show no extra symptom when subsequently inoculated with virulent ones. The mechanism of this acquired immunity is unknown, but it is almost certainly of the non-sterile type, for plants are immune to one strain of a virus only while they contain another strain in a fully active form.¹¹

Although virus-diseased plants do not contain antibodies to the viruses, many plant viruses have now been shown to be good antigens. For when injected into animals they call forth the production of antibodies with which they react specifically. This is further evidence that the absence of antibodies in diseased plants is a result of fundamental differences between plants and animals rather than between the viruses attacking the two types of organisms. Antisera have now been prepared against a number of plant viruses and, as with animal viruses, it is found that different strains of one virus react with each other's antiserum, although they do not react with antisera prepared against unrelated viruses. The serological reactions of plant viruses are not described until the next chapter, but as they play such an important part in the identification and differentiation of virus strains it will be necessary to anticipate some of the work described there.

So many plant viruses have now been found to occur in strains, and some individual viruses in so many different strains, that an entire book would be needed to describe them in detail. Over fifty strains of tobacco mosaic have already been identified. As with this and some other viruses there is strong evidence that new strains are constantly arising, presumably by a process akin to mutation, it is probable that the number of strains that could be isolated is limited by the perseverance of the investigators rather than by the total number of strains. However, most of these variants have been found in the course of experimental work, of which much was expressly designed for the purpose of causing changes, and relatively few strains

of viruses are commonly found in nature. This is no doubt a result of the action of natural selection, for there will be intense competition between related strains. The strains immunise plants against one another, apparently by the presence of one inhibiting the multiplication of others. Therefore, in nature, unless a strain which has newly arisen is more infective than the one from which it has come its chances of survival in any quantity are small. For the more active parent strain will multiply more rapidly and suppress it. The strains found at all commonly in nature, therefore, are those that are most infective, in other words, those most easily transmitted and that multiply and invade plants most rapidly. On the other hand, any new strains arising in experimental work can be isolated from the parent strain and inoculated separately to healthy plants. In such conditions they are freed from the competition of other strains so that variants can be isolated and studied, although they are so poorly infective that they would have little chance of surviving in nature. In the present chapter an attempt is made to discuss the general problem of variations in plant viruses as illustrated by a few examples rather than to describe the individual characteristics of virus strains.

Variations in viruses:—The first suggestions that viruses might alter so that they caused different symptoms are found in the work of CARSNER (1925), JOHNSON (1925) and MCKINNEY (1926, 1929), although viruses now recognised as related strains had previously been described as distinct entities. CARSNER showed that the virus causing sugar beet curly top could be altered by transmission to *Chenopodium murale*, because when returned to beet from this plant it produced only mild symptoms. The change appeared to be a permanent one as repeatedly passing the attenuated virus through susceptible beet failed to increase the severity of the symptoms. However, LACKEY (1932) later showed that the change was reversible and that the virus could be returned to its original condition by passage through *Stellaria media*.

JOHNSON found that tobacco mosaic virus could also be altered so that it produced less severe symptoms. After infecting tobacco plants with the commonly occurring virus he kept them for some days at temperatures of from 35° C to 37° C. The symptoms they showed were milder than those in plants grown at lower temperatures, and they continued mild even when the plants were later kept at more usual temperatures. Evidence that the effect was a real attenuation of the virus and not one on the host plant was obtained by inoculating healthy plants grown continually at lower temperatures with sap from the treated plants, for these also showed only mild symptoms. HOLMES (1934) carried the process of attenuation of tobacco mosaic virus by heat even further. He inoculated pieces of healthy tomato stem with tobacco mosaic virus and then incubated them for 15 days at 34.6° C. They were then macerated and rubbed on to leaves of *N. glutinosa*, where necrotic local lesions were produced. Transfers from these lesions were made to healthy tobacco plants, many of which failed to show any symptoms. Nevertheless, the plants

were infected, because inoculations from them to *N. glutinosa* resulted in the production of large numbers of local lesions. It seems, therefore, that a strain of tobacco mosaic virus had been derived which infects tobacco but fails to cause any visible symptoms. The "masked" strain has now been transmitted serially through tobacco for four years without losing this distinctive characteristic. KUNKEL



FIG. 15. — Tobacco leaf showing symptoms of tobacco mosaic. In addition to the general mild type of mottling, the leaf shows a bright yellow spot. Inoculum taken from this spot causes a general yellow chlorosis. (KUNKEL, L. O., 1934, Ann. Rep. Quebec Soc. Prot. Plants 25-26, 23).

(1937) has obtained an avirulent strain of aster yellows virus by keeping infective leaf-hoppers at 32° C for some days. Vectors kept at this temperature for more than 12 days cease to transmit, but those kept for a shorter time often cause only a mild disease when fed on healthy plants. In further transfers from these plants, using insects kept under normal conditions, this strain consistently produces a milder disease than the normal aster yellows.

McKINNEY demonstrated that tobacco mosaic virus could also change to a form causing more severe symptoms. He called atten-

tion to the fact that plants suffering from tobacco mosaic often showed bright yellow spots in addition to the more general green type of mottle (Fig. 15). He showed also that inoculum taken from these areas and inoculated to healthy plants caused symptoms consisting of a general bright yellow mosaic, quite distinct from the usual symptoms of tobacco mosaic. Although MCKINNEY considered that his tobacco mosaic plants were infected with a mixture of viruses, he suggested that the virus in the yellow areas was not a contaminant in the ordinary sense of the word but that it might be a mutant derived from the form causing the more usual symptoms. He also noted that if plants were inoculated with mixtures of sap from plants with ordinary mosaic and plants with yellow mosaic, symptoms intermediate between the two were obtained, and that the greater the proportion of one virus in the inoculum the less were the symptoms characteristic of the other, and the more slowly they developed.

JENSEN (1933, 1936, 1937) has isolated over fifty strains of tobacco mosaic virus from the yellow spots. Some of these are similar to tomato aucuba mosaic virus, a strain commonly found in nature, but others differ from any strains previously described. JENSEN distinguishes between the various strains by the ease with which they infect and the symptoms they cause in tobacco, tomato, *N. sylvestris* and *N. glutinosa*. All of them at normal temperatures produce only necrotic local lesions on *N. glutinosa*, but some strains produce smaller lesions than others. All cause local lesions on *N. sylvestris*, but with some the lesions are necrotic and with others chlorotic. Also, some give systemic infection and others do not. In tobacco and tomato, the variants show the greatest differences in the severity of the mottling or necrosis and in the degree of distortion and stunting produced. There are also differences in the ease with which they can be transmitted by pricking with needles and in the rapidity with which they move from the entry point in the plant. Making transfers by single pin puncture inoculations some strains gave infection in over fifty per cent of the inoculations, whereas others were transmitted in less than one per cent of the attempts. JENSEN has transmitted some of these variant strains continuously for a period of more than two years and many of them retain their original characteristics. Sometimes, however, especially with strains that move slowly in the infected plants, reversion seems to occur. Then the young leaves of inoculated plants develop either symptoms similar to ordinary tobacco mosaic or else a systemic infection with a rapidly-moving strain, similar to tomato aucuba mosaic virus, producing a general chlorosis.

Similar, though less numerous, variants have been described with cucumber virus 1 and potato virus "X". The commonly occurring cucumber virus 1 causes in tobacco and tomato a rather mild green type of mottling. PRICE (1934) observed that tobacco plants infected with this virus sometimes produced bright yellow spots, like those noticed by MCKINNEY in tobacco mosaic plants. The spots varied in size, shape and intensity of colour, and occurred more frequently in plants growing rapidly than in those growing slowly. PRICE cut out

areas containing these yellow spots, macerated them and rubbed the extract over leaves of healthy tobacco plants. After a few days these developed yellow local lesions and pin-puncture inoculations were made from these local lesions to further healthy tobacco seedlings. Only 14 infections were obtained out of 956 inoculations, but from these, five variants were distinguished. They could be differentiated by the intensity of the mottling and the degree of stunting produced. One strain produced necrotic symptoms in tobacco and moved only slowly from the entry point through the infected plants. Like the slow-moving strains of tobacco mosaic virus isolated by JENSEN, this strain appeared to be unstable, for the young leaves of inoculated plants were always found to contain a more rapidly moving strain producing bright chloroses. The other strains remained constant and produced their characteristic symptoms when transferred serially through numbers of different susceptible species. All the strains isolated from tobacco produced black necrotic local lesions on *Vigna sinensis* (cowpea) and failed to give systemic infection. But among the necrotic lesions produced on cowpea by the normal strain of cucumber virus 1, PRICE noticed two chlorotic lesions. From these he isolated a further strain sharply differing from all the others because it infected cowpea systemically, producing mosaic symptoms. During work on insect transmission of the normal strain of cucumber virus 1, HOGGAN (1935) found occasional plants showing bright yellow mosaics instead of the usual symptoms. From these plants she obtained a strain of the virus which apparently remained unchanged and produced a bright yellow mosaic in tobacco over a period of five years. It is probable that the insects had been feeding on the occasional yellow areas before they were transferred to healthy plants, so effecting the same separation as if these areas had been cut out, macerated and inoculated separately to healthy plants.

Cucumber virus 1 has an extremely wide host range and it is probable that many viruses regarded in the past as distinct entities and given different names are related strains of this virus. PRICE (1935*b*) has shown that southern celery mosaic virus is a strain of cucumber virus 1. It is rather more infectious than the commonly occurring strains and has a slightly different host range, but it protects *Zinnia elegans* against infection with strains of cucumber virus 1. PRICE (1937) has further shown that lily mosaic virus is another strain of this virus. When transferred from *Lilium longiflorum* to tobacco, lily mosaic virus produced necrotic lesions and usually failed to become systemic. On repeated transfers from tobacco to tobacco, however, it behaved like the necrotic, slow moving strain previously isolated (PRICE 1934) from normal cucumber virus 1, and gave rise to a rapidly moving strain which becomes systemic in tobacco, producing the more usual mottling symptoms. AINSWORTH (1938) has obtained similar results with viruses affecting lilies and tulips in England. Again, WHIPPLE and WALKER (1941) have found bean and pea plants in the field infected with strains of cucumber virus 1, although neither this virus nor celery mosaic virus will affect these plants.

What are now recognised as related strains of potato virus "X" were found occurring naturally early in work on potato diseases.

and distinguished by such names as potato mottle and potato ringspot viruses, depending upon the type of symptoms produced in infected tobacco plants. SMITH (1929) suggested that the mottle form gradually increased in virulence when continually passed through tobacco plants, this treatment changing it into the ringspot form. But it is probable that his plants originally contained the two forms, the apparent gradual increase in the severity of symptoms being a result of a gradual increase in the proportion of the ringspot form in the inoculum, which would follow from the selection of the most severely affected leaves to provide inoculum. SALAMAN (1933, 1938) has now distinguished six strains of this virus. All these readily infect tobacco, but one causes no obvious symptoms, two cause faint mottles, one a bright yellow blotchy mottle and the other two cause severe necrotic rings. The individual strains also vary in their reactions in potato, but the symptoms caused by any strain depend greatly on the variety infected. All six strains cause a lethal top-necrosis in the varieties Epicure, Arran Crest and King Edward. In President one strain is carried, three others cause mottles of different severity and the other two cause severe necrotic diseases. In general it can be said that if one strain of a virus causes more severe symptoms in one susceptible plant than another strain it will also cause more severe symptoms in a second susceptible plant. But with all the strains of potato virus "X" this is not so. Thus one strain of this virus produces in tobacco and *Datura* plants an extremely faint, spotty type of mottle, yet in many potato varieties it causes diseases more severe than other strains producing intense ringspot symptoms in tobacco. It is apparent, therefore, that virulence is a term that cannot be applied indiscriminately to viruses or virus strains. It can only be used when a particular virus-host combination is being described.

SALAMAN finds that tobacco plants inoculated from potatoes naturally infected with virus "X" usually contain more than one strain. Tobacco plants infected in this way usually show a mild mottle, with occasional brighter yellow areas or necrotic spots. These symptoms are reproduced in subsequent transfers. On the other hand if the yellow areas are cut out and inoculated separately to healthy tobacco a virus-strain is derived giving a general yellow mottle. Similarly, from the necrotic spots can be derived a strain giving ringspot symptoms. When separated in this way each strain continues to produce its characteristic effects in subsequent transfers. Passage through certain hosts, however, seems to have an attenuating effect on some of the strains virulent to tobacco. When a strain causing ringspot symptoms in tobacco was inoculated to seedling beet, small necrotic rings were formed at the site of inoculation, but no systemic infection occurred. When virus from the local lesions was returned to tobacco it produced only a faint mottle instead of ringspot symptoms.

Variants of different viruses are described so often that it seems to be more exceptional for a virus to exist in a single stable form than for it to exist in many mutant forms, and it is probable that many viruses now thought to be identical will later be found to be related strains, differing in one or more characters. As yet only symptomatology has been at all widely used as a method of differentiating be-

tween strains, and from the few examples given it will be seen that strains have usually been found to differ from one another either in the type or severity of symptoms they cause, or in their host range. Even symptomatology could be more fully used to this end, especially with plants grown in different environmental conditions. MCKINNEY (1935), for example, has described two strains of tobacco mosaic virus which give identical yellow mottles on plants grown at 24° C but which are readily differentiated by the intensity of the symptoms they cause on plants grown at 15° C, and it is to be expected that similar effects will be found with other viruses.

It is also likely that future work will indicate other differences between strains than variations in host reactions and will suggest new methods of differentiating between them. Already, differences between



FIG. 16*a*.—Serologically related strains of a tobacco necrosis virus which consistently crystallise in different ways.—*a*, Thin lozenge-shaped plates formed by one strain and *b* (*next page*) hexagonal prisms formed by the other. $\times 200$.

the insect vectors have been found for strains of potato yellow dwarf (BLACK 1941) and aster yellows viruses (SEVERIN 1934), and at Rothamsted we have been unable to transmit one strain of potato virus "Y" by the aphids that act as vectors of the common strain. Again, one tobacco necrosis virus exists in strains that crystallise differently when precipitated in the same conditions with ammonium sulphate. Of the serologically related strains, which cause identical symptoms in bean and tobacco plants and have many other properties in common, one crystallises in thin, lozenge-shaped plates, the second in hexagonal prisms, and the third has given no recognisable crystals (BAWDEN and PIRIE 1942) (Fig. 16). Viruses differentiated as strains by symptomatology have also been shown to differ slightly in other ways. Aucuba mosaic virus differs from tobacco mosaic virus in its sedimentation constants and X-ray diffraction pattern, and some strains of tobacco mosaic virus differ from others in their contents of particular amino-acids (HOLMES 1941; KNIGHT and STANLEY 1941). There are also

serological differences between strains of tobacco mosaic viruses, for each strain possesses specific antigens in addition to those common to all strains. Except for differences in host reaction, however, the differences found between strains of one virus are few and slight compared with those found between distinct viruses, but they are sufficient to suggest further means of differentiation.

Origin of virus strains:—The viruses grouped together as strains are so alike in all their essential properties that it can be regarded as certain that they have arisen from a common source. And there is a good deal of evidence that many plant viruses are continually giving rise to new variants. It has been shown repeatedly that the reactions of some viruses are quite changed by passing them through a new host or by subjecting infected plants to some unusual environmental

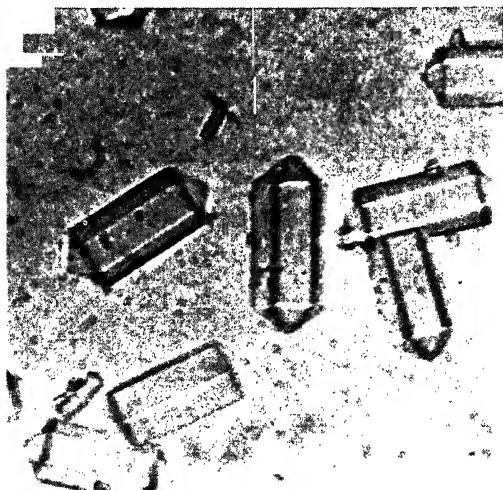


FIG. 16b. — See previous page.

conditions. The change appears to be a sudden one caused by the unusual treatment. As the virus cultures continue to produce their new reactions indefinitely, the change appears to be a permanent one. Thus it is perhaps permissible to refer to the phenomenon as mutation, although the application to viruses of words designed to express changes in organisms reproducing sexually is not without dangers. However, a critical examination of these experiments shows that they cannot be taken as proof that mutation has been caused by the unusual treatment. The change in the reaction may merely mean that the virus-stock used in the experiments was a mixture of strains, and that the unusual treatment may have permitted one strain originally present as a minor constituent to multiply excessively and become a major constituent. Although, of course, the fact that there were two strains present in the virus-stock is evidence that mutation has occurred at some previous time, if not as a result of the treatment.

It is relatively easy to detect the fact that a plant is infected with a mixture of two unrelated viruses. Neither will have any ef-

fect in preventing the other from becoming systemically established. Therefore each will be able to spread throughout the infected plant and produce symptoms, although such a double infection sometimes gives a disease distinct from that caused by either virus when present alone. But when two strains of the same virus are present together in one plant conditions are widely different. Each strain inhibits the development of the other, so that neither is able to become fully systemic, and the symptoms shown by plants infected with such a mixture are usually those characteristic of the strain present in greater quantities. The fact that a mixture is being used, therefore, is difficult to detect unless some selective mechanism is applied. This selective mechanism may be supplied by a new host, or by a new set of environmental conditions, or by deliberately selecting for inoculum portions of infected plants showing different types of symptom.

The attenuation of sugar beet curly top virus by passage through *Chenopodium murale* and the restoration of virulence towards sugar beet by subsequent passage through *Stellaria media*, therefore, has two equally plausible explanations. Passage through *C. murale* may cause a mutation of the virus, and passage through *S. media* may cause the mutated virus to revert back to its original form. On the other hand, curly top virus as it normally occurs may be a mixture of two strains, one virulent to sugar beet and present in larger quantities than the other relatively avirulent one. Each of these strains may multiply equally in sugar beet, so that their relative proportions remain the same and constant symptoms are produced. But in *C. murale* the attenuated strain may multiply the more quickly, so that when the virus-stock is returned to sugar beet the attenuated strain is present in greater quantities and reduced symptoms are caused. In *S. media* the reverse process may occur, the strain causing severe symptoms in sugar beet multiplying more rapidly than the other. Then passage of the attenuated stock of virus through this plant would increase the proportion of the virulent strain in the inoculum, and on returning to sugar beet severe symptoms would again result. Similarly, with potato virus "X" (SALAMAN 1938), the change in reaction of a stock from ringspot to mosaic symptoms by passage through beet may be a result of mutation in the virus particles, or it may be a result of the beet favouring the multiplication of mosaic-producing strains already present in small quantities in the original stock.

The fact that plants suffering from tobacco mosaic normally do contain a mixture of strains is evident from MCKINNEY's work, which shows that the isolated bright yellow areas contain a virus-strain producing symptoms quite distinct from those of tobacco mosaic. For the mere selection of different portions of an infected plant to serve as inoculum could hardly be expected to cause mutation, but only to separate previously existing variants. As infected plants do contain strains producing more severe symptoms than normal tobacco mosaic, it is not unreasonable to assume that they may also contain strains producing less severe symptoms. But as these would not cause any characteristic localised symptoms, as do those causing yellow mosaics

or necroses, their presence would be much more difficult to detect. HOLMES (1934) has shown that the attenuated or masked strains of tobacco mosaic virus multiply more rapidly in plants grown at high temperatures than the strains producing pronounced symptoms. It seems probable, therefore, that the production of attenuated stocks of tobacco mosaic virus by growing recently infected plants at high temperatures is a result of the selection of previously existing avirulent strains, rather than the high temperature actually causing a mutation to avirulence. Because of the presence of large quantities of normal tobacco mosaic virus, a masked strain arising in a plant growing at low temperatures would be prevented from multiplying sufficiently to produce any significant reduction in the severity of the symptoms. But when healthy plants were inoculated with sap from such a plant and then kept at high temperatures, the masked strain would multiply more rapidly than the others. This in its turn would have an inhibiting effect of the multiplication of strains causing symptoms. Thus, it is to be expected that plants inoculated with a minimum of the masked strain and kept at high temperatures would give inoculum consisting largely of the masked strain.

As viruses do exist in strains, and as their reactions can be made to alter, the fact that they mutate can hardly be disputed. The only debatable points would seem to be the frequency of virus mutation and whether a treatment which brings about a change in reaction has actually caused the mutation, or has merely effected a selective action on a previously existing mixture of strains. Proof that a given treatment was causing a mutation could be obtained only if there was certainty that the virus-source used in the experiment was a pure line, that is, contained only one virus strain. Unfortunately, with our present methods of inoculation, it is impossible to be sure that any source of virus contains only one strain. The nearest approximation to a single-spore culture of a fungus that can be obtained with a virus, is a virus-source derived from a single local lesion. There is, however, no knowledge that a local lesion represents infection with a single virus particle. To obtain infection in *N. glutinosa* with tobacco mosaic virus it is necessary to rub the plant with many thousands of virus particles. There will, of course, be a large wastage of inoculum and it is certain that all these particles do not go to the formation of a single lesion, but how many may be required is unknown. In spite of this uncertainty, it is obvious that single local lesions are less likely to contain a mixture of strains than are plants with systemic diseases resulting from numerous separate infections, and in recent work the viruses have been derived from single local lesions. In work with mixtures of aucuba mosaic virus and tobacco mosaic virus KUNKEL (1934) concluded that it was usual for a single local lesion to contain one or other of the viruses and only rarely were both present. When sap at high dilutions is used as inoculum the chances of local lesions containing a mixture of strains will still further be reduced. Yet JENSEN (1936) finds that sources of tobacco mosaic virus derived from single lesions produced by highly diluted sap still give rise to yellow areas containing virus strains causing yellow mosaics.

Some of the strains isolated from these yellow areas are so difficult to transmit, and have such low dilution end-points, that JENSEN considers it highly improbable that they could have been carried as contaminants through numerous single-lesion transfers made with highly diluted sap. This work provides valuable evidence that the strains causing yellow mosaics did arise by mutation in the experimental plants. Some work of MCKINNEY (1935) has also given results strongly indicating that tobacco mosaic virus is continually mutating, and that the strains present in the yellow areas are not to be regarded merely as the separation of a pre-existing mixture into its components. In normal glasshouse conditions *Nicotiana glauca* becomes systemically infected with ordinary tobacco mosaic, but it gives only local lesions with some of the variant strains causing yellow mosaics in tobacco. If inoculum is taken from the young leaves of *N. glauca* and used to infect healthy tobacco plants, these at first show a green type of mosaic. But after some weeks they produce yellow spots from which virus can be derived giving a general yellow mottle in tobacco and which fails to infect *N. glauca* systemically. It is possible that this yellow mosaic virus might have entered *N. glauca* as a contaminant when present in an excess of the ordinary tobacco mosaic virus, although it cannot enter systemically when alone. The result of a further experiment of MCKINNEY seems to make this unlikely and supports the view that the yellow strains must be regarded as newly produced mutants. A strain causing a dark green mosaic has been found by MCKINNEY to be quite stable, and has never given rise to yellow mutants. This becomes systemic in *N. glauca*. When mixtures of this strain and one causing a yellow mosaic were inoculated to *N. glauca*, the young leaves showed a green mosaic. Inoculations from these young leaves to healthy tobacco plants caused the typical dark green mosaic with no yellow spots.

The ordinary strain of tobacco mosaic virus is more infective, *i.e.*, it is more readily transmitted, invades tobacco plants more rapidly and reaches a higher concentration, than most of the other strains that have been derived experimentally. If mixtures of tobacco mosaic virus and other strains are inoculated to the same plant, unless the other strains are greatly in excess they tend to be overrun and their effects suppressed. Yet the experimental variants described give reasonably constant reactions. MCKINNEY (1937) states that the variants he has isolated occasionally give rise to further strains, but that none of them has ever reverted to tobacco mosaic virus. This is strong evidence that his isolations are not mixtures. And if variants of tobacco mosaic virus can be obtained free from tobacco mosaic virus there would seem to be no obvious reason why tobacco mosaic stocks should not be obtained free from variants, except that mutations occur in almost every infected plant.

At first sight the frequency with which variants can be detected would seem to indicate that the detection must be a result of the separation of previously existing strains and not of the production of new mutants. However, if the mutation rate is thought of in terms of virus particles instead of numbers of host plants, the number of variants that can be detected is by no means excessive. A tobacco

plant can be infected by rubbing with about 10^{-10} of a gram of tobacco mosaic virus. Two months after infection at least 50 mgms of virus can be isolated from such a plant. Even if it is assumed that all the virus in the inoculum entered the plant, which it certainly will not, there has been a virus increase of approximately a hundred million times. And such a plant will contain at least a million million (10^{12}) virus particles. In the fruit-fly, *Drosophila melanogaster*, mutation rates have been studied in detail, and in a normal environment a mutation can be expected in from one hundred thousand to a million individuals. Therefore, if the mutation rate in tobacco mosaic virus is of the same order as in *Drosophila*, it is obvious that a large number of variants could be expected in each infected plant.

In view of these figures it is not the number of mutations that have been described, but the constancy of the symptoms produced by tobacco mosaic virus that is surprising. This can probably be explained by the intense competition that goes on in an infected plant between a parent strain and its variants. The variant will only be able to multiply in any quantity and produce its characteristic symptoms if it can get into tissues containing little or none of the parent strain, for the presence of one strain inhibits the increase of another. To become evident it must either be more invasive than the parent strain and be able to move off into newly formed tissues more rapidly, or it must be fortunate enough to find a portion of a leaf not fully infected with the parent strain, as seems to happen in the yellow areas that regularly appear in tobacco mosaic plants. The commonly occurring strain of tobacco mosaic virus is more invasive and occurs in infected plants in greater quantities than any of the experimentally produced variants yet described, and if mixtures of the variants and tobacco mosaic virus are inoculated to healthy plants, the symptoms of tobacco mosaic soon predominate. Thus it is probable that most of the mutants produced from tobacco mosaic virus are unable to compete with the parent strain, and remain undetected because they do not multiply sufficiently to produce any characteristic effects. Most of the mutations that have been produced experimentally in plants and animals have been of the lethal type, rendering the offspring less likely to survive in nature than the parent. This also appears to be true with tobacco mosaic virus. Virus strains most fitted to survive in nature are those easily transmitted, and which rapidly invade and reach a high concentration in the plant without causing too serious a disease. The commonly occurring tobacco mosaic virus possesses all these properties, but the derived mutants mostly lack one or more. The constancy of the symptoms produced by tobacco mosaic virus, in spite of the numerous mutations, can probably be explained on the Darwinian theory of natural selection and survival of the fittest.

It has previously been stated that variants derived from tobacco mosaic virus continue to produce their characteristic reactions in subsequent transfers. However, this is only true to a limited extent, for from these mutants others have been derived (JENSEN 1936; HOLMES 1936; NORVAL 1938). There is some evidence that a charac-

teristic of one strain tends to persist in subsequent mutants. HOLMES has compared the characteristics of mutants causing yellow mosaics derived from a distorting strain and from the masked strain of tobacco mosaic virus. In addition to the difference in symptoms caused by these two strains, they also differ in their ability to invade infected tobacco plants. The distorting strain rapidly spreads into the young leaves of infected tobacco plants, whereas the masked strain does not. HOLMES found that most of the mutants from the distorting strain readily invaded tobacco plants, only a few lacking the characteristic invasive power of the parent strain. On the other hand, none of the mutants derived from the masked strain possessed the invasive power of the distorting strain. NORVAL has obtained somewhat similar results, for he found that twelve strains derived from one causing necrosis of tomato also possessed this property, whereas strains derived from other sources rarely did.

HOLMES (1936) concludes that changes in tobacco mosaic virus to types causing yellow mosaics and to those lacking in invasiveness are independent. He suggests that they represent unit differences in the structure of the virus similar to unit differences in the genetic structure of plants and animals. NORVAL (1938) also compares this virus with genes, and claims to be able to recognise several inheritable "factors" in the virus. SALAMAN (1938) has made similar comparisons with potato virus "X", in which he recognises five distinct "radicles" responsible for the various symptoms produced by the different strains. In view of our ignorance of the chemical nature of genes and of the changes which take place in a chromosome when a mutation occurs, such comparisons, although perhaps inevitable, are not particularly helpful. Indeed, as there is considerably more information about the structure of viruses than about genes, the comparison might be more valuable, though no less dangerous, if made the other way round.

At the present time there would seem to be no valid reason for doubting that the nucleoproteins isolated from virus-infected plants are the viruses themselves. From plants infected with strains of the same virus, nucleoproteins are isolated that are very similar but not identical. It seems, therefore, that when a virus such as tobacco mosaic virus mutates a new protein is formed. Exactly what kind of change occurs is still unknown, but KNIGHT and STANLEY (1941) have found that some strains of tobacco mosaic virus differ from the common strain in the amounts of tyrosine, tryptophane and phenylalanine they contain. Other strains, however, although they cause different symptoms, have the same content of these amino-acids as tobacco mosaic virus itself, so that this type of change is not essential. The results of serological tests show that in addition to the antigens shared by two strains, one or other or both of the strains may contain specific antigens. This implies that some of the constituents of the two strains are chemically identical, whereas others are different. The methods by which viruses produce symptoms in infected plants are also unknown, but the severity of symptoms is not necessarily correlated with virus concentration. The virus content of tobacco plants infected with a strain of potato virus "X" causing severe ringspot

symptoms does not differ significantly from that of plants infected with a strain causing a mild mottle. And the symptoms of aucuba mosaic in tobacco are much more definite than those of tobacco mosaic, although the virus content of plants affected with the latter is higher. It is improbable, therefore, that the symptoms are merely a direct result of virus multiplication in weakening the host plants. It is more probable that the symptoms are a secondary effect of the viruses on the host, produced by active side groups on the virus particles, and that strains produce their specific reactions because they carry specific side groups. Strong evidence for the existence of different groupings in the different strains is supplied by the fact they contain distinct antigens. On this view, the mutation of a virus particle merely involves an alteration in its active groups. For the loss, or the acquisition, of one or more active groups, or an alteration in the chemical structure of side groups, would result in the production of a strain causing different symptoms, but otherwise resembling the original virus.

Acquired immunity:—Two types of behaviour in virus infected plants have been described under the term acquired immunity. The first is the gradual reduction in the severity of symptoms that occurs in some diseases, so that after a time infected plants may be almost indistinguishable from uninfected plants, although they no longer develop the severe disease if again inoculated with the same virus. The second is the protection afforded to a plant against a virulent strain of a virus by previous infection with an avirulent strain. The use of the term acquired immunity for either of these reactions has been criticised adversely by many workers, and there is little doubt that the term is being used to describe phenomena different from those in animals for which it was coined. In animals acquired immunity can be either active or passive, but both are usually of the sterile type, the originally susceptible animal being immune because of the presence of antibodies to the pathogen. In active acquired immunity the animal has produced the antibodies itself as a result of direct contact with the pathogen whereas in passive acquired immunity the antibodies have been received as such from the blood of another animal. With virus diseases of plants the sequence of events superficially resembles that with diseases of animals, but the resistance acquired by recovery from an acute disease, as well as the protection afforded against virulent strains of a virus by earlier infection with an avirulent strain, is of the non-sterile type and persists only as long as the virus is present in the plant tissues in an active state. The recovery is rarely complete and the effect of avirulent viruses is usually obvious, so that plants are in the main rendered resistant to serious diseases only by continually suffering mild diseases. With animals acquired immunity usually implies complete resistance to a pathogen whereas with plants it is resistance to a set of symptoms. The resistance in plants is sometimes incomplete, for, if infection is not fully systemic, tissues uninvaded can still be infected by re-inoculation. Thus it would undoubtedly be preferable to use some term other than acquired immunity for the phenomena in plants;

but until one has been agreed upon it seems unlikely that its use can cause much confusion provided the differences between plants and animals are emphasised.

The first type of acquired immunity was described by WINGARD (1928), who showed that a number of different kinds of plants infected with tobacco ringspot virus do not remain severely affected indefinitely. About three days after infection the inoculated leaves show necrotic local lesions. Some days later severe necrotic rings and spots appear on the young uninoculated leaves. After about a fortnight, however, new leaves are produced on which lesions may either fail to develop or develop only slightly. New leaves produced after this stage may look quite normal, or show a faint grey mottle instead of the severe necroses. After a few weeks the older leaves that developed necroses mature and die, leaving a plant that may be impossible to distinguish from one that has never been attacked.

WINGARD found that suckers or cuttings taken from recovered plants gave rise to plants that also looked healthy. However, the apparently normal leaves, on both recovered plants and on cuttings from recovered plants, failed to develop any ringspot or other symptoms when reinoculated with sap from severely diseased leaves. On the other hand, sap taken from the leaves of recovered plants regularly caused ringspot symptoms when rubbed over the leaves of healthy plants. WINGARD suggested that infected plants acquire an immunity to the disease, a view that has since been vigorously supported by PRICE (1932, 1936*a* and *b*). Other workers have objected to the use of the term acquired immunity to describe this phenomenon, preferring to call it acquired tolerance, to call the recovered plants carriers and to look upon the effect as "masking" of symptoms. It is true that the recovered plants are not immune to the virus. PRICE has propagated recovered plants through ten generations by means of cuttings, and the progeny contained virus which caused typical ringspot symptoms when rubbed on to normal tobacco plants. All the plants propagated from recovered plants failed to develop ringspot lesions when rubbed with infective sap, whereas similar cuttings from normal plants were severely affected. The recovered plants can be regarded as carriers of the virus, or to have acquired a tolerance to the virus, but the fact that they have developed an immunity to the ringspot disease can hardly be questioned. The infectivity of sap from leaves on recovered plants is less than one-fifth of that from leaves with ringspot symptoms (PRICE 1936*a*). STANLEY (1939) isolated virus from both kinds of infected leaves and found no difference in properties. From necrotic leaves, however, the yield of virus was about 1 part in 80,000 and from symptomless leaves 1 part in 500,000.

VALLEAU (1941) states that the degree of recovery varies with environmental conditions, but is never complete. Plants grown at 20° C show definite symptoms, though not of the ringspot type, and only at 26° C do infected plants grow like normal ones. Even then, recovered plants have much pollen sterility and produce few seeds. MCKINNEY (1941) also finds incomplete recovery and states that recovered plants yield less than healthy ones. Most plants raised

from the seeds of recovered plants are free from virus and develop typical ringspot when inoculated with sap from infected plants. A few examples of seed-transmission of some strains of the virus, however, have been noticed, and all plants inheriting the virus have been found to be immune to the ringspot disease (VALLEAU 1932). Immunity, therefore, does not necessitate having the disease but depends upon the presence of virus in the tissues. On first entering susceptible tissues the virus causes a severe reaction, but when systemically distributed throughout the plants it multiplies without producing much serious effect. As the virus in recovered plants is identical with that in necrotic leaves, the difference must lie in the host and not the virus.

VALLEAU (1941) suggests that the first severe phase of the disease is due to the infection of mature tissues and that the less severe symptoms in recovered plants are a result of tissues being infected in the embryonic state. As the virus content of leaves on recovered plants is less than that of necrotic leaves, this indicates that infection of young tissues alters the metabolism of the host so that the virus is prevented from multiplying sufficiently to cause its severe effects.

WALLACE (1938) has recently reported a similar phenomenon in tobacco plants infected with sugar beet curly top virus. At first the plants show severe symptoms, but later the apical growth or new side shoots show slight symptoms, sometimes being almost normal in appearance. These recovered shoots, and plants propagated from them by cuttings, still contain the virus. They show no fresh symptoms when reinoculated with the same strain or with other strains of curly top virus. A suggestion that something similar to passive acquired immunity in animals may occur with tomatoes infected with curly top virus has recently been made by WALLACE (1940). He states that virus transmitted by leaf-hoppers from recovered to healthy tobacco plants gives the typical severe symptoms, whereas transmission by grafting gives only mild symptoms. He also states that plants such as tomatoes, which normally do not recover from curly top virus in the same way as tobacco, acquire some resistance when grafted with scions from recovered tobacco plants. WALLACE suggests that these results indicate protective substances in the recovered plants which were transferred by grafting, but the possibility that the plants were infected with mixtures of strains, the virulent ones being more readily transmitted by leaf-hoppers, does not seem to have been excluded. Recovery, though less pronounced than with ringspot or curly top, also occurs with some other diseases. Virulent strains of potato virus "X" at first cause severe necrotic rings and spots in tobacco plants, but after a few weeks the infected plants often show only mild mosaics or no definite symptoms. Also, many potato varieties in the first year of infection with potato virus "Y" react with a severe necrotic disease, but in the second and subsequent years they show few necroses, the main symptoms being those of a crinkle.

PRICE (1936b) has described four tobacco ringspot diseases from the severe symptoms of which plants regularly recover and are sub-

sequently immune. Plants recovered from one of these may also be immune from others, though not all. For example, plants recovered from yellow ringspot also resist ordinary ringspot and green ringspot, but plants recovered from ringspot No. 2 develop typical symptoms when inoculated with any of the others. The sequence of events in plants infected with tobacco ringspot viruses, the severe disease soon after infection followed by recovery and lasting immunity, closely parallels that in many virus diseases of animals. The only significant difference is the apparent absence of antibodies from the recovered plants and the consequent persistence of the virus in them. However, there are a few diseases of animals in which the animals are stated to contain active virus long after they have ceased to show symptoms and have become immune. Examples of these are the salivary gland disease of guinea pigs (COLE and KUTTNER 1926), and infectious anemia of horses (KOCK 1924), and it is possible that animals recovered from these diseases possess a non-sterile type of immunity similar to that of *Nicotiana* species recovered from ringspot.

The second type of acquired immunity was indicated by the work of MCKINNEY (1929), but was first clearly demonstrated by THUNG (1931) with tobacco mosaic virus and by SALAMAN (1933) with potato virus "X". From a plant suffering from tobacco mosaic, THUNG isolated a variant causing a "white mosaic" when inoculated to healthy tobacco plants. When inoculated to plants already infected with tobacco mosaic virus it produced no change of symptoms. THUNG suggested that the presence of one of these viruses in a cell exerted an antagonistic effect on the other, so preventing its entrance. SALAMAN obtained similar results with potato virus "X". He found that tobacco plants fully infected with strains of this virus causing mild mottles or no appreciable symptoms did not develop any further symptoms when inoculated with virulent strains producing in healthy tobacco plants severe mosaics or necrotic rings. Since then a similar phenomenon has been described with these and with other viruses by numerous different workers. All have found the protecting effect to be quite specific. That is, plants infected with one strain of tobacco mosaic will be protected against further infection with other strains of this virus, but will still be susceptible to other viruses such as potato virus "X" and cucumber virus 1. Similarly, plants infected with one strain of potato virus "X" will be protected against other strains of this virus but not against infection with tobacco mosaic virus.

How this protective effect is obtained is still uncertain. If two strains of the same virus are inoculated together into a healthy plant, both multiply. However, they often tend to settle into separate parts of the same leaf, so that by selecting for inoculum portions of a leaf showing symptoms characteristic of each strain it is possible to regain what appear to be pure cultures of the strains. Also, when extracts from plants infected with different strains are mixed and allowed to stand, neither strain is inactivated. The protection, therefore, is clearly not a result of a direct neutralising effect of one strain on another. It seems probable that there is a limiting amount of any

particular virus that a plant can contain, and if one strain has already multiplied to this amount a second is unable to multiply. As a plant fully infected with one virus is still susceptible to other unrelated viruses, this implies that related viruses either multiply at the same sites, or utilise the same materials, whereas unrelated viruses multiply at different sites, or utilise different materials.

The degree of protection afforded by the presence of one strain in a plant to subsequent infection with another depends upon a number of factors. It varies with the length of time the plant has been infected, with the method of infection, the species of the host plant,

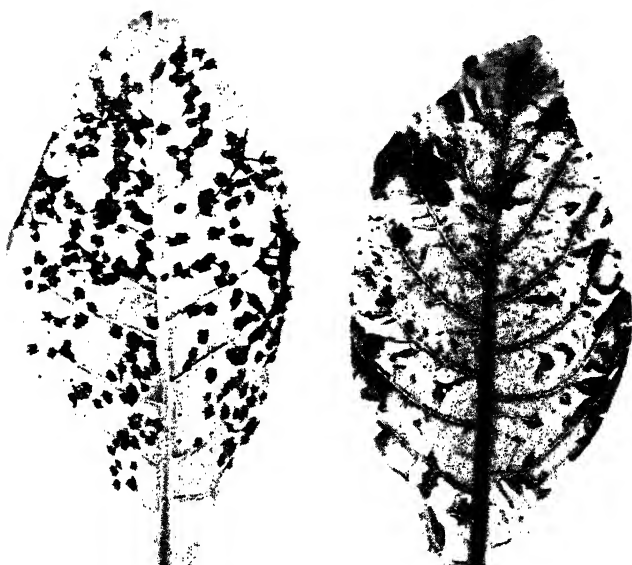


FIG. 17. — Two leaves of *Nicotiana sylvestris* as they appeared 5 days after both had been inoculated with tomato aucuba mosaic virus. The leaf on the left was healthy whereas that on the right was suffering from tobacco mosaic at the time of inoculation with aucuba mosaic virus. The local lesions appear only on the leaf which was healthy at the time of inoculation. (KUNKEL, L. O., 1934, *Phytopath.* 24, 437).

the age of the plant, and relative infectivities of the strains used. Variations in any of these conditions may be expected to affect the virus content of different plant tissues, which is closely correlated with the degree of protection.

This correlation is clearly indicated by KUNKEL's (1934) experiments with tomato aucuba mosaic virus and tobacco mosaic virus, strains sharply differentiated by their reaction on *N. sylvestris*, the former causing necrotic local lesions and the latter a systemic mottling. Infection with tobacco mosaic virus does not immediately render the plants immune from aucuba mosaic virus, but with increasing length of time after inoculation with the former *N. sylvestris* become increasingly resistant to the latter (Fig. 17). It is evident that the immunity develops after, and as a result of, infection with tobacco mosaic virus. The immunity can be produced by directly inoculating

a leaf, or by a leaf becoming infected as a result of the systemic spread of the virus. The length of time required to produce the immunity depends upon the age of the leaf and the method of infection. If the leaf is mature and infected by direct inoculation of tobacco mosaic virus, it becomes immune in a few days, but the immunity is restricted to the actual areas inoculated. Also, the immunity is rarely complete unless a concentrated inoculum of tobacco mosaic virus is used. If the leaf is infected by systemic spread of the virus, the immune areas closely approximate to those showing symptoms. Young actively developing leaves soon show symptoms and become immune. On the other hand, leaves too old to show symptoms of tobacco mosaic, in which the virus content increases slowly, remain susceptible to aucuba mosaic virus for six weeks after the plants are inoculated. The correlation between the degree of protection afforded and the virus content of the plant is clearly shown by the quantitative studies made by SADASIVAN (1940). He rubbed leaves of tobacco and *N. sylvestris* with tobacco mosaic virus and tested their virus content and their susceptibility to aucuba mosaic virus at various intervals. As the content of tobacco mosaic virus increased, so did the number of lesions produced by aucuba mosaic virus decrease, until after eight days it produced none.

With potato virus "X" similar results have been obtained (SALAMAN 1938). Five days after inoculation with an avirulent strain, tobacco seedlings are quite immune to virulent strains. But those reinoculated with the latter at from one to five days after infection with the avirulent strain show necroses of decreasing severity. The protection is again restricted to areas in which the first strain has become fully established. If tobacco plants systemically infected with a mottling strain of this virus are rubbed all over with inoculum containing a virulent strain, some necrotic local lesions are obtained but no general necrotic disease. The areas bearing the local lesions when isolated are found to contain only the virulent strain, whereas leaf tissue between the necrotic lesions contains only the mottling strain (BAWDEN 1934). Similarly, mottled plants reinoculated with the virulent strain occasionally develop necrotic rings on uninoculated leaves. When isolated and used as inoculum these also are found to contain the virulent strain, but not the other. Thus, it seems clear that the presence of one strain in a plant neither inactivates another nor prevents its entry. Unless the second strain alights upon tissues in which the first has not become fully established, however, it is unable to multiply sufficiently to cause symptoms.

Potatoes infected with one strain of virus "X" are usually quite immune to the effects of others when reinoculated with them. But if the second strain is introduced by means of a graft, protection is rarely complete (BAWDEN 1934; KOHLER 1935). The young side shoots of the stocks develop symptoms less severe than those characteristic of the more virulent strain alone, the effect being similar to that obtained by inoculating plants with a mixture of the strains. KOHLER states that different strains of this virus give varying degrees of protection against infection with other strains. One strain that causes a mosaic in tobacco will protect that plant against other strains causing

mosaics, but will only partially protect against strains causing ringspot. SALAMAN's results do not support this, but it is possible that the strains used by the two workers are distinct.

It is comparatively easy to show that avirulent virus-strains protect plants against the effects of virulent ones, but to show the reverse is not so easy unless the avirulent strain has a characteristic reaction on some other host. It is generally accepted that the protection is reciprocal. That is, if an avirulent strain immunises a plant against a virulent one, the virulent strain will also immunise against the avirulent. Where this has been tested it has been found to be true, though the degree of protection afforded by different strains may vary. An invasive strain that multiplies rapidly would be expected to give complete protection against a strain that is weakly invasive and multiplies slowly. On the other hand, the presence of this second strain in a plant would be unlikely to immunise against the first, although it would give a certain amount of protection.

MCKINNEY (1935) finds that tobacco plants suffering from tobacco mosaic show no change of symptoms when reinoculated with strains causing yellow mosaic. But when plants suffering from yellow mosaic are reinoculated with tobacco mosaic virus, the symptoms on subsequently formed leaves become less severe than those of typical yellow mosaic. They are typical of those caused by a mixture of the two virus-strains. If the plants are kept for long periods, or if the tops are cut off so that new side shoots are produced, the young leaves show the typical green mottling of tobacco mosaic with only a few scattered yellow spots. Similarly, tobacco plants infected with potato virus "X" have never been found to become infected with virus "D" when reinoculated, whereas potato virus "D" does not protect completely against potato virus "X" (BAWDEN 1934). PRICE (1936*b*) finds that plants recovered from yellow ringspot are quite immune to ringspot No. 1 and to green ringspot. On the other hand, tobacco plants recovered from ringspot No. 1 or from green ringspot, although immune from one another, are only partially protected from yellow ringspot. All these results clearly show that the protective effect is not in preventing the entry of the second strain into infected plants, but merely the prevention of its multiplication. The efficiency of one strain in preventing the multiplication of another appears to depend upon its ability to multiply and occupy the plant fully. For tobacco plants infected with tobacco mosaic virus have a higher virus content than those infected with strains causing yellow mosaics, and those infected with potato virus "X" have a higher content than those infected with potato virus "D". Nothing is known about the relative virus contents of plants infected with ringspot No. 1 and yellow ringspot. But again it is possible that the greater protective effect of the latter results from the fact that it reaches a higher virus concentration, or is more invasive. It will be noticed that this type of acquired immunity is quite different from any that has been reported with diseases of animals, for, as far as the writer is aware, there are no examples known in which an animal continually has one disease and is thereby protected against another.

Although it is generally true that the protection acquired by an

infected plant is specific and restricted to serologically related strains of the infecting virus, an unusual interaction has been found between potato virus "Y" and severe etch virus (BAWDEN and KASSANIS 1941). Tobacco plants infected with severe etch virus apparently resist infection with potato virus "Y", whereas plants infected with potato virus "Y" are susceptible to severe etch virus. If the two viruses are inoculated simultaneously into healthy plants, these develop symptoms identical with those inoculated with severe etch virus alone, and the sap from such plants appears to contain no virus "Y". Similarly, when plants infected with virus "Y" are reinoculated with severe etch virus they develop typical symptoms of severe etch and their sap ceases to react with antisera to potato virus "Y". Thus it seems that severe etch virus can prevent the multiplication of virus "Y" and supplant it, either completely or in part, even in plants in which it has become well established. Other viruses, such as potato "X" and tobacco mosaic, have no such effect on potato virus "Y", and severe etch virus also has no effect on the multiplication of these in susceptible plants. There is, therefore, considerable specificity even in this reaction and as the two viruses concerned are alike in many of their properties, they may be related though not sufficiently so to contain common antigens.

The presence of one virus in a plant may affect the results of inoculation with another in any of several ways. If the two are related strains, the multiplication of the second may be inhibited so that protection is complete and there is no change of symptoms; or protection may be only partial, with slight change in symptoms as a result. If the two viruses are not serologically related, an effect similar to that described with potato virus "Y" and severe etch virus may result, or the two viruses may interact in some other way to produce symptoms which may differ from those produced by either virus alone. Tobacco plants recovered from ringspot are readily infected with tobacco mosaic virus, but they show milder symptoms than plants infected with tobacco mosaic virus alone (PRICE 1932). By contrast, tobacco or tomato plants infected with potato virus "X" become acutely necrotic if they become infected with tobacco mosaic virus. Strains of virus "X" which produce no visible local lesions in healthy tobacco leaves cause necrotic spots when rubbed over leaves already infected with tobacco mosaic virus. Other pairs of viruses do not seem to interact; tomato plants suffering from spotted wilt, for example, are said to develop a typical yellow mottle when inoculated with aucuba mosaic virus (CALDWELL 1935). Thus the inoculation of a second virus may have no effect or it may produce typical or atypical symptoms; if the latter, the symptoms may be either more or less severe, or even of a different type, than the second virus would produce alone.

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Chapter VII

SEROLOGICAL REACTIONS OF PLANT VIRUSES

Methods:— It is impossible in this chapter to attempt to give any details of the theory and practice of serology. For a full account of general serology the reader is referred to TOPLEY and WILSON (1937) and MARRACK (1938), and for plant serology to CHESTER (1937*a*).

TOPLEY and WILSON define an antigen as any substance which, when introduced parenterally into the animal tissues, stimulates the production of an antibody, and which, when mixed with that antibody, reacts with it in some observable way. An antibody is defined as any substance which makes its appearance in the blood serum or body fluids of an animal, in response to the stimulus provided by the parenteral introduction of an antigen into the tissues. The term parenteral is used to emphasise the fact that the antigen must reach the animal tissues in an unaltered state. It will be noted that two properties are attributed to the antigen; it must have the power of stimulating the production of an antibody, and it must react specifically with that antibody. Both of these properties are essential, as there are substances which react specifically with antibodies, without possessing the power of stimulating their formation. Such incomplete antigens are known as haptens.

Substances with widely different properties can act as antigens. But antigens are most frequently large molecules or molecular aggregates, and they are usually, though not invariably, composed wholly or partly of protein. At their surfaces they present so-called determinant groups, which may be of varied chemical structure and are not necessarily chemically complex. These groups determine the specificity of the serological reactions. How the antibodies are produced in response to the injection of antigens is quite unknown. Those which have had their properties examined have been found to resemble the serum globulins, but to be modified in such a way that they unite specifically with one or other of the determinant groups carried by the antigen. The union of the antibody and antigen is followed by a number of reactions which can conveniently be observed *in vitro*. The most obvious is the formation of insoluble material which settles out as a visible precipitate. This precipitin reaction has been mainly used in work on plant viruses, but complement fixation, neutralisation of infectivity, and anaphylactic shock have also been demonstrated.

Precipitin reaction:— Precipitation occurs when an antigen in solution is mixed with its antiserum in the presence of electrolytes, provided that the concentration of antigen and antibody, and the experimental conditions, are suitably arranged. The rate at which

precipitation occurs can be greatly increased by any treatment that increases the frequency of impact between antigen and antibody, or between the first formed floccules of the antigen-antibody complex; for example, by increasing the concentration of the reagents, by heating the mixtures, or by ensuring continuous mixing either by mechanical shaking or by convection currents. The rate of precipitation is also increased if antibody and antigen are present in optimal combining proportions, for the ratio of one reagent to the other greatly affects both the speed and completeness of precipitation. Indeed, if there is a great excess of one, especially of antigen, precipitation may be quite inhibited. In any precipitation experiment, therefore, it is essential that tests should be made at a number of antigen/antibody ratios, to ensure that precipitation is not being inhibited by an excess of one or the other.

The following method has been found most successful for carrying out careful precipitation tests with plant viruses. The tests are made in thick-walled, 7 mm glass tubes, 1 cc. of diluted antiserum being mixed with 1 cc. of antigen solution, all dilutions being made in 0.85% NaCl solution. Immediately the two are mixed, the tubes are placed in a water-bath at from 45° C to 50° C, with fluid columns half immersed so that convection currents keep the mixtures continuously agitated. The water-bath has a glass front and is illuminated from behind so that the tubes can be observed without being moved. In some tests the antiserum is used at a constant dilution in all the tubes and the dilution of the antigen varied; in others the antigen is kept constant and the dilution of antiserum varied.

However, this method can only be used satisfactorily with clear, heat-stable antigen preparations. The difficulties of preparing such stable preparations of plant antigens has been long recognised (WELLS and OSBORN 1911). The sap of many plants, whether healthy or virus infected, after clarification by centrifugation or by filtration, frequently forms spontaneous precipitates after standing for some time at room temperature. This precipitation occurs even more rapidly in a heated water-bath. In experiments in which clarified sap is used as the test antigen, therefore, a rigid system of controls, in which the sap is mixed with normal serum and saline, must be used to differentiate between the specific and the spontaneous precipitates. This difficulty can be overcome, in part at least, by subjecting the infective sap to treatments which destroy the unstable components. If the virus is heat-stable, the sap can be heated for a few minutes at about 60° C. This causes the separation of a bulky green precipitate, readily removed by filtration or centrifugation, and leaves a clear, stable supernatant fluid. If the virus is heat-labile, the stability of the clarified sap can be increased by freezing the leaves at low temperatures before extracting the sap, as many of the unstable components of sap are rendered insoluble by freezing. Alternatively, the sap can be clarified by the addition of Na_2HPO_4 and centrifuging, the virus then being precipitated from the supernatant fluid with ammonium sulphate and redissolved in water. Provided that the precipitation tests are not carried out at too high temperatures, antigens prepared in this

way are sufficiently free from spontaneous precipitates to give reliable results.

Complement fixation:—Although complement fixation reactions are often more sensitive than precipitation as an indication of antigen-antibody union, they have not yet been widely used in the study of plant viruses. This is, no doubt, because the technique is both difficult and laborious. The result of the union of antigen and antibody is observed indirectly by its effect in inhibiting another reaction. The reaction inhibited is the lysis (dissolution) of sheep blood corpuscles by the serum of rabbits immunised by injection with sheep corpuscles. Two constituents are necessary for the lysis of the corpuscles. One is the antibody produced in the rabbit in response to the injection of corpuscles. The other is a thermo-labile, nonspecific constituent of normal serum, known as complement. When the antigen and antibody being tested unite, complement is used up or fixed. The amount of fixation varies with the degree of reaction between antigen and antibody, and is measured by observing whether or not there is still sufficient free complement to allow the lysis of the sheep-blood corpuscles in the presence of anti-sheep-cell rabbit serum. This serum, known as the haemolytic amboceptor, is freed from its complement by heating to 56° C, the standardised complement used in fixation experiments being provided by fresh guinea pig serum.

The experiments are carried out in two parts. First, the antigen and antibody under test are mixed and complement added. The mixtures are allowed to stand for one hour, for fixation of the complement to take place. Then, washed sheep corpuscles and the haemolytic amboceptor are added. After mixing, the fluids are kept at 37° C and frequent readings made on the degree of lysis of the blood cells. The reactions taking place are summarised below:

1. Haemolytic amboceptor + Complement + Corpuscles = Lysis
2. Antigen + Antibody + Complement = Fixation
3. 2 + Haemolytic amboceptor + Corpuscles = No lysis.

It will be noticed that the lysis of the corpuscles has nothing to do with the union of the antigen and antibody under examination. It is purely an indicator reaction, showing whether or not the antigen and antibody have united and fixed the complement. As in the precipitation tests, the complement fixation experiments should be carried out over a range of antigen and antibody dilutions, for zones of optimal reaction are obtained, excess of one or the other greatly inhibiting the fixation of complement (*See Fig. 18*).

Neutralisation of infectivity, I:—The union of a toxic antigen with its antibody is usually accompanied by a loss of toxicity. Similarly, the mixing of viruses with their antisera is accompanied by a loss of infectivity. At first sight this would seem to be the simplest serological reaction of viruses to test, for it is apparently only necessary to mix infective sap with antiserum and determine the result on infectivity to show whether a reaction has occurred. Unfortunately, it is not quite so simple, for normal sera and heterologous antisera also

greatly inhibit infectivity. The specific effect of homologous sera can be taken as the difference between the reduction in infectivity caused by heterologous and other sera, but this is far from exact as the un-specific neutralisation varies considerably with different sera. Therefore to ensure that a specific serological reaction has occurred and brought about the reduction in infectivity, it is necessary to do accurate infectivity tests, using adequate controls with normal serum and saline.

Anaphylaxis:— In the anaphylactic reaction, the union of antibody and antigen is indicated by changes in the animal tissues. In the living animal, there may be more or less violent spasmodic muscular contractions, inflammation of tissues, or other abnormal effects, often resulting in death. *In vitro*, the most sensitive form of anaphylactic test is the Schultz-Dale (DALE 1913) technique. Virgin guinea pigs are immunised by injection of the antigen. After three weeks, they are killed and the two horns of the uterus are dissected out. To each end of the horns is attached a thread. The uterine horn is then placed in an aerated Ringer's solution, kept at 37°C. The lower end is tied rigidly to the bath, the upper end is attached to a kymograph needle. A small quantity of the antigen is then introduced into the Ringer's solution, and a positive reaction is shown by a rapid contraction of the uterine horn. This is followed by a slow relaxation, both contraction and relaxation being recorded on the kymograph. A guinea pig can be sensitised against a number of different antigens at once, and the uterine horns will react successively with each antigen. But each horn will react only once with one antigen, as one reaction completely desensitises it. CHESTER (1936a) has used this method with great success in differentiating between the antigenic constituents of healthy and virus-infected plants. Care must be used in the interpretation of the results of this technique with plant antigens. Extracts of plants, especially of members of the *Solanaceae*, contain substances causing non-specific muscular contractions. These can be distinguished from the specific serological reactions as they are given by the uterine horns of unsensitised guinea pigs; also, the muscles will react to the non-specific toxins several times in succession, provided they are washed and allowed to relax after each contraction. CHESTER has found that these toxic substances can be removed completely from plant extracts by a few hours' dialysis against Ringer's solution.

Preparation of antisera:— Two methods of immunising animals are customarily used, either intravenous or intraperitoneal injections of the antigen. Intravenous injections often produce a high antibody concentration more rapidly, but intraperitoneal injections are less likely to produce serious reactions in the animals. For this reason, when crude plant extracts are used as immunising antigens, intraperitoneal injections are to be preferred. With highly purified virus preparations, however, antisera precipitating at dilutions of greater than 1 in 1,000 have been prepared by a single intravenous injection of 1 mgm. This is undoubtedly the best method of producing anti-viral

serum, as the serum is also free from antibodies to any normal plant constituents. But only a few viruses have yet been purified, and the antisera to most viruses have been produced using crude or clarified infective sap as antigens.

It is usual to give a series of from 5 to 10 intraperitoneal injections at intervals of about 5 days. From 2 to 4 ccs of infective sap are injected each time. After the last injection, the animals are allowed a rest period of about 10 days before being bled. The number of injections, and the volume of sap injected, necessary to produce an antiserum varies with different viruses, depending upon their stability and their concentration in the sap. It is much less with stable viruses occurring in relatively high concentrations, such as tobacco mosaic and potato "X", than with viruses such as potato "Y". The blood drawn from the immunised animal is allowed to stand overnight to clot, when the clear serum is separated from the clot by centrifugation. Provided that it is kept sterile, the antiserum remains serologically active for many years. Most workers have used rabbits for the production of antisera, but CHESTER (1936*a*) has used guinea pigs and NEWTON and EDWARDS (1936) have produced antisera to potato virus "X" in chickens.

Extracts of virus-infected plants are much more effective as producers of precipitating antibodies than extracts of healthy plants. Nevertheless, to ensure that reactions obtained with infective extracts are specific, antisera prepared against crude sap should be fully absorbed with sap from healthy plants of the same species before being tested against infective sap. This is done by mixing the serum with from 2 to 5 times its volume of normal plant sap, allowing the mixture to stand overnight, and then removing the precipitate by centrifugation. If the supernatant fluid still gives any reaction with normal plant sap, the absorption should be repeated.

Alternatively, a method described by DOUNIN and POPOVA (1938) can be used to provide antisera free from antibodies to normal plant constituents. An antiserum is first formed using sap from healthy plants as the immunising antigen. Infective sap of the same species is then allowed to react fully with this antiserum, so precipitating any antigenic constituents of normal plants. These are removed by centrifugation, and the supernatant fluid when injected into animals produces antiserum giving no precipitin reactions with normal sap.

The antigenicity of plant viruses:—The first indication that plant viruses might be antigenic is found in the work of DVORAK (1927), who prepared antisera separately against healthy and mottled Green Mountain potatoes. Precipitin tests showed that extracts of both potatoes had antigens in common, but each antiserum gave a higher titre with its homologous than with its heterologous antigen. That is, the antiserum prepared against sap from mottled potatoes reacted more strongly with sap from mottled potatoes than with sap from healthy potatoes, and *vice versa*. By precipitating the sap with ammonium sulphate, DVORAK showed that the difference lay in the globulin fraction of the sap, and the explanation offered was that the

disease had altered the precipitability of the globulins. As it is now known that all Green Mountain potatoes, whether obviously diseased or healthy looking, are virus infected, these tests were probably distinguishing between plants infected with one virus and plants infected with more than one virus, rather than between virus-free and virus-infected plants. This fact would also explain the higher titre obtained by DVORAK for antiserum prepared against "healthy" potatoes than that obtained by subsequent workers using virus-free plants as controls.

Proof that a virus-infected plant contains a specific antigen was provided by PURDY BEALE (1928, 1929, 1931). She produced antisera separately against extracts of healthy tobacco and tobacco infected with tobacco mosaic virus. Some antigenic substances were found to be common to both extracts, but after antiserum to infective sap was completely absorbed with healthy sap it still precipitated and fixed complement strongly with infective sap. Extracts of plants other than tobacco infected with tobacco mosaic virus reacted with the tobacco mosaic virus antiserum, whereas extracts of plants infected with other viruses did not.

GRATIA (1933*a*, 1933*b*) first showed that plants infected with different viruses contain different specific antigens. He prepared antisera separately against extracts of plants infected with a potato mosaic virus and with tobacco mosaic virus. Each antiserum precipitated strongly with the extract used to produce it, but not with the other. BIRKELAND (1934) obtained similar results, but further showed that extracts from plants infected with viruses believed to be related strains contained antigens in common, in addition to those also found in healthy plants. He prepared sera against sap from plants infected separately with tobacco mosaic virus and attenuated tobacco mosaic virus, two strains of spot necrosis (probably a mixture of potato viruses "X" and "Y") and potato ringspot, and cucumber virus 1. The serum prepared against one strain of tobacco mosaic virus reacted with extracts of plants infected with the other, but not with the other viruses. Similarly, serum prepared against either strain of spot necrosis precipitated with the other and with potato ringspot, but not with tobacco mosaic virus or cucumber virus 1. And serum prepared against cucumber virus 1 reacted only with sap from plants infected with this virus. Similar results have since been found with other viruses, extracts of plants infected with viruses known to be related strains having precipitating antigens in common, whereas those of plants infected with distinct viruses are not serologically related.

Although these tests showed conclusively that virus-infected plants contain specific antigens, and that the serological reactions could be used with certainty as a method of identifying the virus with which a plant was infected, they gave no indication as to the nature of the specific antigens. The workers generally believed that the infecting viruses were behaving as antigens, but it was equally possible that the specific antigens were merely products of the host plant produced as a result of virus-infection. A good deal of work has been done in an attempt to decide between the two possibilities, and the evidence is

now overwhelmingly in favour of the view that the specific antigens are the viruses themselves.

MATSUMOTO and SOMAZAWA (1932, 1933) examined tobacco mosaic plants in some detail by serological methods, and found that the specific antigen was always associated with the presence of the virus, as indicated by infectivity tests. If virus was present, so was the antigen regardless of whether or not symptoms could be seen. BIRKELAND (1934) showed that the passage of sap from tobacco mosaic plants through filters retaining the virus also removed the specific precipitating antigen. SPOONER and BAWDEN (1935) obtained similar results with potato virus "X"; filtrates through collodion membranes fixed complement and precipitated with antiserum if they were infective, but not if they were virus-free. Experiments were also made to determine the effect of inactivating the viruses upon their serological reactions. In general it was found that treatments destroying infectivity also destroyed the serological reactions, the antigen specific to a virus infected plant possessing the stability characteristic of the infecting virus. CHESTER (1935*b*) showed that when tobacco mosaic virus, tobacco ringspot virus and potato viruses "X" and "Y" are inactivated by heating, or by progressive strengths of KMnO_4 , AgNO_3 and chloramine-T, the serological reactions are retained as long as the viruses are present and active; they diminish in strength in direct proportion to the loss of infectivity and they disappear when the viruses cease to be infective. Similarly, when potato virus "X" is inactivated by the proteolytic enzymes, trypsin, pepsin and papain, the reduction in serological activity is directly proportional to the reduction in infectivity (BAWDEN and PRIE 1936). Some lesions are occasionally obtained from preparations which give no precipitate with antiserum, but this merely indicates the greater sensitivity of the infectivity test. A similar effect is obtained with diluted preparations, the precipitation end point being at a smaller dilution than the infection end point.

However, there are some treatments that render the viruses non-infective without affecting their ability to react with antiserum. These are treating with formaldehyde, dilute hydrogen peroxide, or nitrous acid, and irradiation with X-rays or ultra-violet light (BAWDEN 1935; STANLEY 1936; BAWDEN and PRIE 1936, 1937*a*, 1937*b*, 1938*a*, 1938*b*). It is probable that these treatments leave the antigenicity of the virus unimpaired, although only preparations of potato virus "X" have been shown to be fully antigenic, *i.e.*, capable of stimulating the formation of antibodies *in vivo* in addition to reacting with them *in vitro*, after such inactivation. BAWDEN, PRIE, and SPOONER (1936) showed that partially purified preparations lost their infectivity if treated with nitrous acid. The method used was to adjust the preparation to pH 4 with HCl, cool to 0°C , and then add sufficient of a strong solution of NaNO_2 , previously adjusted to pH 4 with acetic acid, to give a final concentration of 1.5% NaNO_2 in the treated preparations. After half an hour at 0°C , the preparation was adjusted to pH 7 and dialysed to remove the excess nitrite. Preparations treated in this manner were non-infective but reacted with antiserum to active virus preparations. Also, when injected intra-

Table 5:

Potato virus "X". Precipitation test with active virus, virus inactivated with nitrous acid, and healthy plant preparation, and their respective antisera.

Serum prepared against —	Dilution	Antigens		
		Active virus suspension 1/20 (1-hour reading)	Nitrite-treated inactive virus suspension 1/20 (1-hour reading)	Healthy sap preparation 1/20 (24-hour reading)
Active virus suspension	1/4	++++	++++	—
	1/8	++++	++++	—
	1/16	+++	+++	—
	1/32	++	++	—
	1/64	+	+	—
	1/128	—	—	—
	0	—	—	—
	1/4	++++	++++	—
Nitrite-treated inactive virus suspension	1/8	++++	++++	—
	1/16	++++	++++	—
	1/32	+++	+++	—
	1/64	++	++	—
	1/128	+	+	—
	0	—	—	—
	1/4	—	—	—
	1/8	—	—	—
Healthy sap preparation (all 24-hour readings)	1/16	—	—	—
	1/32	—	—	—
	1/64	—	—	—
	1/128	—	—	—
	0	—	—	—
	1/4	—	—	—
	1/8	—	—	—
	1/16	—	—	—

+ signs indicate the degree of precipitation; ++++ a bulky precipitate; + a small precipitate; — no precipitation.

venously into rabbits they caused the production of an antiserum indistinguishable from that produced by fully active virus preparations. Both antisera fixed complement and precipitated with active virus preparations, but not with the sap of healthy plants, and both were equally effective in neutralising the infectivity of potato virus "X" when mixed with it *in vitro*. The results of complement fixation experiments and precipitation experiments with antisera prepared against active virus, virus inactivated by nitrous acid, and healthy plant extracts, with the three antigens are shown in Fig. 18 and Table 5.

At first sight the inactivation of viruses by nitrous acid, formaldehyde, and other treatments, without affecting the serological reactions, suggests that the specific antigens are not the viruses. However, it is known that some of these treatments can also destroy the pathogenicity of some bacteria without destroying their antigenicity. Also, it will be shown in later chapters that these treatments have no effect on other characteristic properties of the viruses. It seems that they cause sufficient changes in the viruses to render them non-infective, without denaturing them or causing any changes in the antigenically active groupings.

If the antigens specific to virus-infected plants are merely diseased

host products, they must differ remarkably from normal plant constituents in their antigenicity. Animals injected with tobacco mosaic sap soon produce antisera precipitating, or fixing complement, with their homologous antigen at dilutions of 1/1,000 or greater. By contrast, some workers have failed to demonstrate any specific precipitating antibodies in the serum of animals injected with healthy tobacco sap. Where these have been demonstrated the titre of the serum has always been low, and the antigens responsible for them are readily removed from the antigen specific to the infected plants by precipitation with acid or salts. It will be noticed in Fig. 18 and Table 5, that no reactions were obtained using healthy plant extracts (or the serum prepared against them) treated in the same way as extracts from infected plants. On the other hand, CHESTER (1936a) has shown that some constituents of healthy solanaceous plants are active antigenically in producing anaphylactic shock, as indicated by the Schultz-Dale technique, whereas the antigen specific to tobacco mosaic plants is quite inactive in this way. This was shown in the following manner. Guinea pigs immunised with either healthy or tobacco mosaic sap give strong anaphylaxis with both. But if the uterine horns of pigs immunised with infective sap are allowed to react with healthy sap they become quite desensitised and give no further reaction when tested against infective sap or against concentrated virus preparations. Hence the antigens responsible for this anaphylactic reaction must be entirely constituents of normal plants. In sharp contrast to the results of the anaphylaxis absorption experiments, the serum of guinea pigs immunised against infective sap still precipitated and fixed complement strongly after it had been allowed to react fully with healthy plant sap. In the same animal, therefore, some normal plant constituents can be highly active as producers of anaphylaxis but relatively inert as producers of precipitating antibodies, while the antigen specific to infected plants produces a good precipitating antiserum but fails to cause anaphylaxis in the excised uterine horns. CHESTER suggests that the different behaviour of the normal plant constituents and the virus in the Schultz-Dale reaction may be a result of their different sizes. The first are probably small particles or molecules which readily diffuse into the uterine muscles, whereas the virus particles are too large to do this. SEASTONE, LORING and CHESTER (1937) have shown that tobacco mosaic virus is not inert as a producer of anaphylaxis. They confirmed CHESTER's earlier results that the virus did not give the Schultz-Dale reaction, but found that the injection of infective sap, or of purified virus, into living guinea pigs immunised against infective sap and then completely desensitised with healthy plant sap frequently produced fatal anaphylactic shock. Thus it appears that the virus does produce antibodies with which it can unite to cause anaphylaxis, but the union is prevented in the excised uterus and occurs only in the living animal. BEALE and SEEGAL (1941) have also found that purified tobacco mosaic virus produces anaphylactic shock *in vivo*, although the virus and normal tobacco proteins were much less anaphylactogenic than animal proteins.

If the antigens specific to infected plants were merely host reaction

products, it also becomes necessary to assume that every susceptible species can produce exactly the same product. Healthy plants of tobacco and phlox contain no demonstrable amounts of serologically related substances, but both are susceptible to tobacco mosaic virus. When they are fully infected, both contain large quantities of a common antigen, for antiserum prepared against one reacts strongly with sap from the other. It is, of course, possible that in addition to multiplying itself in these two unrelated plants, the virus also causes the production of this active antigen, but at present there is no reason to doubt that this common antigen is the virus itself.

The fact that antiserum prepared against infective sap has a specific quantitative effect in neutralising the infectivity of the particular

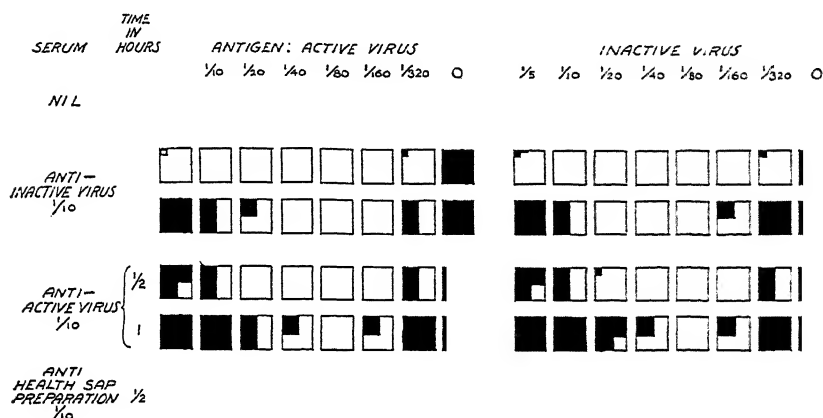


FIG. 18.—*Potato virus "X"*. Complement fixation by active virus preparations and preparations inactivated with nitrous acid, with antisera prepared against each, and against a healthy plant preparation. The haemolytic amboceptor was used at three times its minimal strength with the dose of complement used. Fixation was allowed to proceed for 1 hour before the sheep-blood corpuscles were added. The degree of haemolysis is indicated by the blackening of the squares: a totally black square indicates a tube in which haemolysis was complete, a white square one in which there was no haemolysis. Note the optimal zone of fixation.

virus when mixed with it *in vitro* again suggests a close relationship between the antigen and the virus. This is further indicated by the fact that the strength of the serological reactions of infective sap is directly proportional to its virus content as measured by infectivity tests (BEALE 1934; BAWDEN 1935). However, by far the strongest evidence in favour of the viruses themselves being the specific antigens comes from the work on the purification of the viruses. The purest virus preparations are the most active serologically, *i.e.* with increasing purity less solid material is necessary to give a visible reaction with antiserum. The highly purified preparations, consisting of liquid crystalline and crystalline nucleoproteins, that have recently been made have serological titres of the same order as other antigens that have been carefully purified. There is no doubt that these nucleoproteins are the antigens specific to virus-infected plants. Although it cannot be proved that these are the viruses (they may be host-reaction products contaminated with active virus), all the evidence suggests

that they are. And for the present there would seem to be no reason to doubt that the viruses themselves are the exceedingly active antigens present in infective sap.

Neutralisation of infectivity, II: — MULVANIA (1926) found that the addition of fresh, normal rabbit serum to tobacco mosaic sap caused a decrease in the infectivity. Other workers have confirmed this, but have found that the serum of rabbits immunised with tobacco mosaic sap is more effective than normal serum in inhibiting infectivity. (PURDY BEALE 1928; MATSUMOTO 1930; SILBERSCHMIDT 1933). Similar results have also been obtained with potato virus "X" (SPOONER and BAWDEN 1935). The most detailed work on inhibition of infectivity has been done by CHESTER (1934).

CHESTER prepared antisera to tobacco mosaic virus, tobacco ring-spot virus, and cucumber virus 1, and found that each serum and normal rabbit serum reduced the infectivity of preparations of any of the three viruses when mixed with them. This property was also shared by other fluids containing proteins, such as healthy tobacco sap, milk and solutions of ovalbumin. In addition to this non-specific effect, each antiserum was found to have a specific effect in neutralising the infectivity of the particular virus preparation used in making the antiserum, but not in reducing the infectivity of the other two viruses. For example, normal rabbit serum, tobacco ring-spot virus antiserum, and cucumber virus 1 antiserum, all reduced the infectivity of tobacco mosaic sap equally, but tobacco mosaic virus antiserum reduced it more (Fig. 19). The reduction in infectivity is a quantitative effect; if the amount of virus present in a preparation is doubled, the amount of antiserum must also be doubled to produce the same inhibition.

CHESTER (1934) states that the non-specific and the specific effects of antiserum are produced differently. He claims that the non-specific effect is one of decreasing the susceptibility of the host plant, whereas the specific one is a direct effect on the virus. Two facts were given as evidence for this claim. Firstly, the full effect of normal serum occurred immediately the serum and virus were mixed, whereas the effect of antiserum increased with the length of time the mixtures were allowed to stand before being inoculated to plants. Secondly, the percentage reduction in infectivity caused by the addition of a given amount of normal serum was approximately the same at all virus dilutions, whereas the specific effect of antiserum was greater at high dilutions than at low. The effect of various antisera on the infectivity of tobacco mosaic sap is shown in Fig. 19. That the homologous antiserum has the greatest effect in reducing infectivity cannot be disputed. But that the specific effect of tobacco mosaic virus antiserum is qualitatively as well as quantitatively different from the effect of normal serum cannot be taken as proved. YODEN, BEALE and GUTHRIE (1935) have fitted CHESTER's results to the dilution curve $y = N(1 - e^{-ax})$, and find that they are better suited by a change in a than by a change in N . That is to say, they indicate a reduction in the concentration of active virus rather than a reduction in the susceptibility of the host plant, indicating that the normal

serum is acting directly on the virus. CALDWELL (1936) in a series of dilution experiments with normal serum also found the effect to be on the virus and not on the host.

It seems probable that both the specific and the non-specific effect of antiserum in reducing infectivity is a direct result of the serum on the virus. What happens is unknown, but it is probable that the virus forms non-infective complexes by uniting with the serum proteins. The union with the antibody is a firmer one than with the ordinary serum proteins. But union with the antibody does not de-

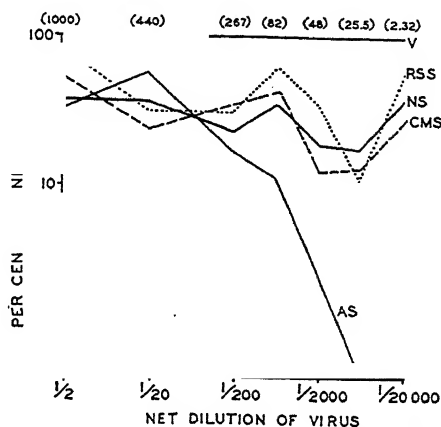


FIG. 19. — *The inactivation of tobacco mosaic virus with various sera.* Inhibition of infectivity of tobacco mosaic virus at various dilutions by normal serum (NS), tobacco ringspot virus antiserum (RSS), cucumber virus 1 antiserum (CMS), and tobacco mosaic virus antiserum (AS), all at dilutions of 1/200. Infectivity expressed as percentage of serum-free control (V); numbers in parenthesis above each point in the dilution scale are the actual number of lesions formed by the control on bean leaves. (CHESTER, K. S., 1934, *Phytopath.* 24, 1180).

stroy the virus. The infectivity is merely neutralised, and the effect is in part reversible. If mixtures of virus and normal serum, or mixtures of virus and antiserum, are diluted largely with saline they regain some of their infectivity. Rather less dilution is needed with the normal serum than with the antiserum. This recovery of infectivity is shown in Table 6. A sample of tobacco mosaic virus was divided into three:

Table 6:
Effect of dilution on the infectivity of mixtures of tobacco mosaic virus and sera

Dilution	Average number of lesions per leaf		
	Saline control	Normal serum	Tobacco mosaic virus antiserum
1/1	240	24	2
1/5	210	40	4
1/25	52	38	9
1/125	29	18	9
1/625	13	5	6

to one portion was added an equal volume of 0.85% NaCl solution, to the second an equal volume of normal serum at a dilution of 1/10, and to the third an equal volume of tobacco mosaic virus antiserum at a dilution of 1/10. After thorough mixing, the fluids were allowed to stand for one hour, when they were diluted with saline and their infectivities tested at different dilutions. It will be seen that the addition of both sera caused a great reduction in infectivity, but when the mixtures were diluted some was regained. This can probably be attributed to dilution causing a dissociation of non-infective com-

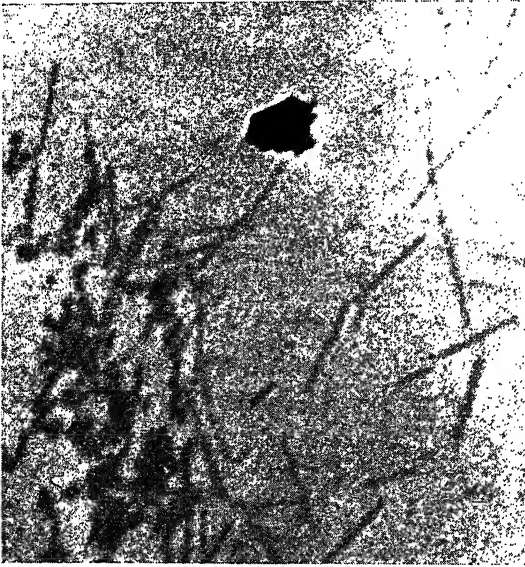


FIG. 20.—A mixture of tobacco mosaic and bushy stunt viruses treated with antiserum to bushy stunt virus and photographed by the electron microscope. The particles of bushy stunt virus are agglutinated but the rod-shaped particles of tobacco mosaic virus are unaffected. $\times 45,000$. (ANDERSON, T. F. and STANLEY, W. M., 1941, *J. Biol. Chem.* 139, 335).

plexes formed when virus and serum are present in greater concentrations.

This recovery of infectivity can be demonstrated even more strikingly in other ways. CHESTER (1936c) found that non-infective and serologically-inactive precipitates, produced by mixing tobacco mosaic sap and its antiserum, regained some of their infectivity if incubated with pepsin at pH values at which the enzyme is proteolytically active. After neutralisation, the treated mixtures also precipitated specifically with fresh antiserum, but not with fresh virus. Hence, the enzyme had hydrolysed some of the antibodies in the precipitate, so liberating active virus. BAWDEN and PRIE (1937a) obtained similar results with purified tobacco mosaic virus. The mixture of virus and antiserum containing the amorphous precipitate was almost non-infective. After centrifuging, the precipitate was incubated with pepsin at pH 3. It soon developed a sheen, and after sufficient incubation the virus was regained with all its characteristic liquid crystalline properties, in

addition to its normal infectivity and serological activity. With the less stable potato virus "X" CHESTER (1936c) has recovered active antibody from the virus-antiserum precipitates. When the precipitates were acidified they broke down, the antibody being liberated and the virus denatured. After being centrifuged and neutralised, such treated mixtures precipitated strongly with fresh virus. From these results it is obvious that, although union between viruses and their antibodies

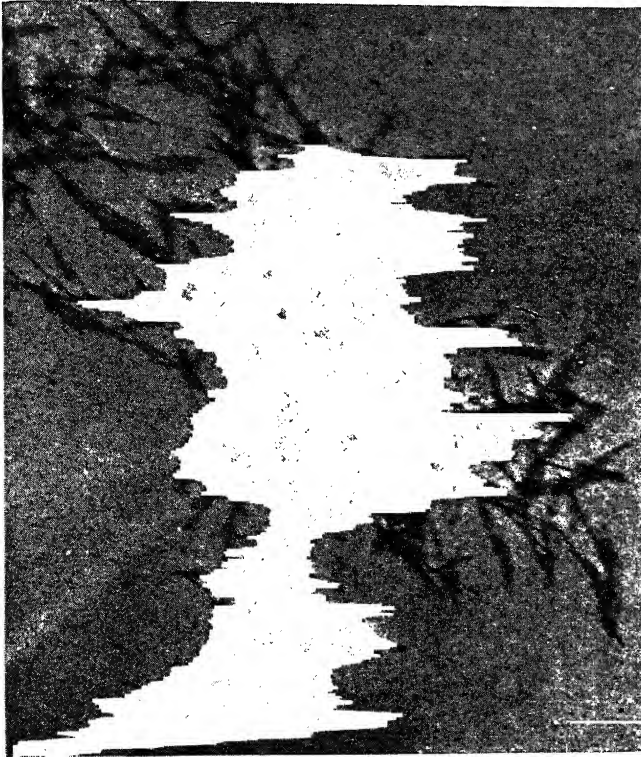


FIG. 21. — A mixture of tobacco mosaic and bushy stunt viruses treated with antiserum to tobacco mosaic virus and photographed by the electron microscope. The rod-shaped particles of tobacco mosaic virus are agglutinated but the smaller particles of bushy stunt virus are unaffected. $\times 17,000$. (ANDERSON, T. F. and STANLEY, W. M., 1941, *J. Biol. Chem.* 139, 339).

results in the loss of the characteristic properties of both, neither are destroyed by the union. For by appropriate treatments both can be recovered with all their original properties.

Specificity of serological reactions:—Because of their specificity, one of the greatest uses of serological reactions in plant virus work is in the rapid identification of viruses. A serum prepared against one virus will react with the sap of any plant infected with that virus, but not with the sap of any plant infected with a different virus. Figures 20 and 21 illustrate this specificity of the precipitin reaction. A mixture of tobacco mosaic and bushy stunt viruses was

divided and one part treated with antiserum to tobacco mosaic virus and the other with serum to bushy stunt virus. The mixtures were then photographed by means of the electron microscope. It will be seen that in the presence of tobacco mosaic virus antiserum the small bushy stunt particles are unaffected, whereas in the presence of the bushy stunt virus antiserum the small particles have been agglutinated and the rod-shaped tobacco mosaic virus particles remain unaffected.

To identify a virus by means of its host range or symptomatology, even if aided by studying its stability *in vitro*, takes some weeks and the result is often uncertain. On the other hand, if a reaction can be obtained with an antiserum to a known virus, the virus can be identified with certainty in a few minutes. However, the identification is not exact, as the serological reactions are only group specific. If the sap of a plant gives a positive reaction with tobacco mosaic virus antiserum, it is certainly infected with tobacco mosaic virus. But the straightforward serological tests usually give no indication as to the exact strain of tobacco mosaic virus present. Most workers who have found that viruses such as potato virus "X", cucumber virus 1 and tobacco mosaic virus do not react with each other's antisera, so that they can be readily identified by their specific serological reactions, have been unable to distinguish between the strains of these individual viruses by precipitation, complement fixation, or virus-neutralisation tests. In general, the strains of one virus react in an essentially similar manner with their homologous and the heterologous antisera. This is clearly shown in Table 10 where the results are given of testing two strains of potato virus "X" separately, and in mixtures of different proportions, against an antiserum to one of them.

However, the fact that one virus strain reacts with antiserum prepared against another merely shows that the two strains contain common antigens, but does not prove that they are antigenically identical. If each virus is not a simple unit antigen, but carries a number of different determinant groups, then the antiserum will also contain a number of different antibodies, each reacting specifically with its particular determinant group. Two viruses which have one or more determinant groups in common will be precipitated equally by each other's antiserum. But if, in addition to the common antigenic groups, each strain contains specific groups, then their effects on their homologous and heterologous antisera will be different. Each strain will react fully with its homologous antiserum, removing all the antibodies from it. It will remove from its heterologous antiserum, however, only those antibodies for which it has determinant groups, the others specific to the groups peculiar to the second strain being unaffected. The presence of such specific antibodies is shown by the formation of a precipitate when the virus strain used for producing the serum is added to a sample that has been allowed to react fully with a second strain. Using small letters to represent determinant antigenic groupings and capital letters for the corresponding antibodies, three strains of the same virus and their antisera might be represented by the following formulae:

Antigenic groupings

Strain 1.	a, b, c, d, e.
Strain 2.	c, d, e, f, g, h.
Strain 3.	a, b, g, h, j, k.

Antibodies

Antiserum 1.	A, B, C, D, E.
Antiserum 2.	C, D, E, F, G, H.
Antiserum 3.	A, B, G, H, J, K.

As each antiserum contains some antibodies capable of uniting with antigens of each strain, mixture of any of the strains with any of the antisera will cause the precipitation of the virus. But the effect on the antiserum will depend upon the particular strain mixed with it. Thus, if antiserum 1 is allowed to react fully with strain 1 all five antibodies will be bound, or absorbed, and can be removed in the precipitate, so that the supernatant will have no precipitating action when mixed with further virus. If, on the other hand, antiserum 1 is fully absorbed with strain 2, the supernatant will still contain antibodies A, B. Therefore, it will give no further precipitation when more strain 2 is added, but will precipitate with both strains 1 and 3. Similarly, if it is fully absorbed with strain 3, antibodies A, B, will be removed, but C, D, E, will remain, and a further precipitate will be obtained on the addition of either strain 1 or strain 2. Using this method of analysing the antibody composition of the sera, it has been possible to show that tobacco mosaic virus and potato virus "X" are complex antigens, and to differentiate between the individual strains of these viruses.

CHESTER (1936b), using clarified infective sap as his antigen preparations, found that antiserum to tobacco mosaic virus could be absorbed fully with tomato aucuba mosaic virus so that it gave no further precipitate with this virus, although it still precipitated with tobacco mosaic virus. He also found that some of the strains isolated experimentally by JENSEN (1933) were serologically identical with the naturally occurring aucuba mosaic virus, and others with the parent tobacco mosaic virus. By serum absorption experiments CHESTER also distinguished between three strains of potato virus "X", and indicates the serological differences detected by the following antigenic formulae:

Mottle strain	a, b, c.
Masked mottle strain . . .	a, b, d.
Ringspot strain	a, d, e.

Using purified preparations of the viruses as antigen preparations, BAWDEN and PIRIE (1937b) have made cross-absorption experiments with various strains of tobacco mosaic virus and their antisera. The results obtained are summarised in Table 7. In addition to the antigens common to the three recognised strains of tobacco mosaic virus, tobacco mosaic and aucuba mosaic viruses contain an antigenic fraction not present in enation mosaic virus, aucuba mosaic and enation mosaic viruses contain a fraction not present in tobacco mosaic virus, and enation mosaic virus contains a fraction not present in either of the others. If each antigenic fraction be represented by a letter, the differences are most simply expressed by the formulae:

Tobacco mosaic virus . . .	w, x.
Aucuba mosaic virus . . .	w, x, y.
Enation mosaic virus . . .	w, y, z.

There is, however, no reason to believe that each antigenic difference detected is a single antigen, and it is probable that by using more strains in such experiments greater numbers of antigens would be detected. In these cross-absorption tests were included cucumber viruses 3 and 4. These were found to be serologically related to tobacco mosaic virus, although they will not infect tobacco, and no hosts are known susceptible to these and to tobacco mosaic virus. Serologically they differ from tobacco mosaic virus much more than the strains such as aucuba mosaic and enation mosaic viruses which have similar host ranges and can be differentiated only because they cause different symptoms. The cucumber viruses have only a few antigens in common with tobacco mosaic virus, whereas aucuba and enation mosaic viruses have many. This fact is clearly indicated by the ability of sera fully absorbed with their heterologous antigens to precipitate their homologous antigens. After antiserum prepared against any of the strains of tobacco mosaic virus has reacted fully with cucumber virus 3, its precipitation with its homologous antigen is only slightly less than that of untreated serum, the optimal precipitation point shifting only slightly. Similarly, the reaction of cucumber virus 3 antiserum with cucumber virus 3 is little altered by absorption with tobacco mosaic virus, showing that only a few antibodies have been removed by the absorption. On the other hand, when antiserum to one strain of tobacco mosaic virus is fully absorbed with another strain, its precipitation with the strain used for immunisation is greatly affected, the optimal precipitation point being greatly shifted, showing that many antibodies have been removed. It seems, therefore, that the serological reactions, in addition to being of value in identifying viruses, can also be adapted to differentiate between strains of the same virus, and to indicate the degree of relationship between different strains.

That the antigenic fraction *w*, common to the three strains of tobacco mosaic virus tested, is not a single antigen, is indicated by the tests with cucumber virus 3. After absorption with either enation mosaic or aucuba mosaic viruses, tobacco mosaic virus antiserum loses its power of precipitating cucumber virus 3. Therefore, the antigens shared by cucumber virus 3 and tobacco mosaic virus are in the common fraction *w*. Removing the whole of *w* by absorption with enation or aucuba mosaic viruses greatly reduces the power of the serum to precipitate. As removal of the antigens common to cucumber virus 3 and tobacco mosaic virus only slightly affect the precipitating power of the serum, it is apparent that these antigens form only a minor part of the fraction *w*.

It has been stated that straightforward precipitin tests with virus strains and their homologous and heterologous antisera usually indicate no differences in the serological reactions of the strains. The relationships between cucumber virus 3 and tobacco mosaic virus are such that the two can be clearly distinguished by their different behaviour when titrated against both antisera. The serological titre given by preparations of either virus, *i.e.*, the greatest dilution at which a visible precipitate is obtained, are nearly independent of the serum used, but the range of antigen dilution over which precipitation

Table 7:
Summarised Results of Cross-absorption Experiments

Precipitation tests with absorbed sera and antigens						
Antiserum absorbed	Antigen used for absorption	Antigen				
		Tobacco mosaic virus	Aucuba mosaic virus	Enation mosaic virus	Cucumber virus 3	Cucumber virus 4
Tobacco mosaic virus	T.M.V.					
	A.M.V.			—		
	E.M.V.	++				
	C.V. 3	++++		++++		
	C.V. 4	++++		++++		
Aucuba mosaic virus	T.M.V.		++	++		
	A.M.V.		—			
	E.M.V.	++	++			
	C.V. 3	++++	++++	++++		
	C.V. 4	++++	++++	++++		
Enation mosaic virus	T.M.V.		+	++		
	A.M.V.			+		
	E.M.V.			—		
	C.V. 3	++++	++++	++++		
	C.V. 4	++++	++++	++++		
Cucumber virus 3	T.M.V.				++++	++++
	A.M.V.				++++	++++
	E.M.V.				++++	++++
	C.V. 3					

— indicates that there is no precipitation.
+ signs indicate the degree of precipitation at the optimum.

occurs varies widely with the two sera. When cucumber virus 3 is titrated against a constant amount of antiserum to any of the three strains of tobacco mosaic virus, there is a large zone in the region of antigen excess where no precipitation occurs. Similarly, when any of the three strains of tobacco mosaic virus used are titrated against a constant amount of cucumber virus 3 antiserum, there are large zones of non-precipitation in the antigen excess region. By contrast, when cucumber 3 is titrated against a constant amount of its homologous antiserum, or when any of the three strains of tobacco mosaic virus are titrated against their own or each other's antiserum, such zones of non-precipitation are much smaller, occurring only where the antigen is very much more concentrated. The results in which tobacco mosaic virus and cucumber virus 3 preparations were titrated against constant amounts of four antisera are shown in Table 8. It will be seen that there are differences in the range of precipitation obtained with the antisera to the three strains of tobacco mosaic virus, but these are insignificant in comparison with the large differences between these and the cucumber virus 3 antiserum.

These results can be best explained by postulating quantitative as well as qualitative differences in the antigenic constitution of cucumber virus 3 and tobacco mosaic virus. Most of the antigenic groupings in cucumber virus 3 are absent from tobacco mosaic virus, and *vice versa*. But it seems probable that they have two common antigens, the sum total of the two in each virus being of the same order, although tobacco mosaic virus contains a preponderance of one and cucumber virus 3 a preponderance of the other. If the common antigens are called p and q, then tobacco mosaic virus will contain many p antigenic groups and only a few q, whereas cucumber virus 3 will contain only a few p and many q. With such different quantitative compositions it is apparent that the antiserum prepared against one of these viruses will contain only a few antibodies to the major antigen of the other, which will therefore have to be diluted greatly before the antibody/antigen ratio is optimal for precipitation. KNIGHT and STANLEY (1941) have found that a virus affecting plantain reacts with tobacco mosaic virus antiserum in much the same way as do cucumber viruses 3 and 4. This virus from plantain and the cucumber viruses were found to differ widely from tobacco mosaic virus in their content of some amino-acids, whereas other strains of tobacco mosaic virus, such as aucuba mosaic virus, which react strongly with tobacco mosaic virus antiserum, did not differ from tobacco mosaic virus in this way. These differences in the quantities of individual amino-acids may represent the quantitative differences between the common antigens postulated in the previous paragraph, but it is unlikely that they could also explain the large qualitative antigenic differences there are between tobacco mosaic virus and cucumber viruses 3 and 4.

In using cross-absorption tests for identifying virus strains so-called "mirror tests" must be made before two strains can be shown to be antigenically identical. Antisera must be prepared separately against each, and it must be shown that neither reacts with its homologous antiserum after absorption with the other strain. The necessity for

the mirror tests is indicated in the results given in Table 7 with tobacco mosaic and aucuba viruses. After absorption with aucuba mosaic virus, antiserum against tobacco mosaic virus gives no further reaction with tobacco mosaic virus. It might be assumed, therefore, that the two are identical. However, after absorption with tobacco mosaic virus, antiserum to aucuba mosaic virus still reacts with aucuba mosaic virus. The two are not identical, but aucuba mosaic virus contains all the antigens of tobacco mosaic virus and at least one additional specific antigen.

It has been stated above that if the sap of a plant infected with an unknown virus reacts with a serum prepared against a known virus then the unknown can be identified as a strain of the known. The converse is not necessarily true, for the absence of a reaction does not prove the unknown virus to be serologically distinct from the known. This can only be proved by preparing antisera against both the known and the unknown viruses, and showing that neither reacts with the other's antiserum in the same conditions as it reacts with its own. Such reciprocal tests are necessary as the virus content of sap infected with different strains may vary widely. And the absence of a reaction with one strain may merely mean that the sap contains insufficient virus to produce a visible precipitate. Therefore, it is necessary to show that expressed sap contains sufficient antigen to produce a visible precipitate, by obtaining a reaction with homologous antiserum, before a negative result can be accepted as proof of lack of serological relationships.

In general, viruses immunising plants against one another also react with each others' antisera. However, PRICE (1935, 1937) has shown that plants infected with celery mosaic and lily mosaic viruses are protected against further infection with cucumber virus 1, whereas CHESTER (1937*c*) obtained no precipitin reaction when sap from plants infected with these two viruses was mixed with cucumber virus 1 antiserum. This may be an example of viruses immunising plants against one another without being serologically related. On the other hand, as no antisera were prepared against celery mosaic or lily mosaic viruses, it is equally possible that they are serologically related to cucumber virus 1, but that the sap of plants infected with them contains too little virus to give a visible reaction. This quantitative effect is clearly shown with strains of *Hyoscyamus* virus 3. During work on insect-transmission, WATSON (1938) isolated a variant producing only mild symptoms in tobacco, and the sap of infected plants produced fewer lesions than that of plants infected with the virulent strain. When tested against antiserum to the original strain, either using crude sap (CHESTER's field method, 1937*b*) or clarified sap, extracts of plants infected with the attenuated strain give no precipitin reaction. But if the virus is concentrated, by precipitation with ammonium sulphate and re-solution in a small volume of water, it readily precipitates with antiserum to the virulent strain.

The serological titres of purified plant virus preparations range from 10^{-6} to 10^{-7} (BAWDEN and PIRIE 1937*a* and *b*, 1938*a* and *b*). It is obvious then that unless infective sap contains from 10^{-6} to 10^{-7} gms of virus per cc. it will not contain sufficient to produce a visible

Table 8:
Precipitation of Tobacco Mosaic Virus and Cucumber Virus 3 with Different Antisera

1. Tobacco Mosaic Virus as Antigen

Antiserum	Time	Dilution of antigen (1/1 = 1 mg. per cc.)					
		1/1	1/4	1/16	1/64	1/256	1/1024
Tobacco mosaic virus	2 min.	++	++	+	—	—	—
	2 hr.	++++	++++	++++	+++	++	+
	24 "	++++	++++	++++	++++	+++	++
Aucuba mosaic virus	2 min.	++	++	+	—	—	—
	2 hr.	++++	++++	++++	+++	++	+
	24 "	++++	++++	++++	++++	+++	++
Enation mosaic virus	2 min.	—	++	+	—	—	—
	2 hr.	—	++++	++++	+++	++	+
	24 "	—	++++	++++	++++	+++	++
Cucumber virus 3	2 "	—	—	—	—	+	+
	24 "	—	—	—	+	++	++

2. Cucumber Virus 3 as Antigen

Antiserum	Time	Dilution of antigen (1/1 = 1 mg. per cc.)					
		1/1	1/4	1/16	1/64	1/256	1/1024
Tobacco mosaic virus	1 hr.	—	—	—	+	++	—
	24 "	—	—	—	++	+++	++
Aucuba mosaic virus	2 "	—	—	—	—	+	—
	24 "	—	—	—	—	++	++
Enation mosaic virus	2 "	—	—	—	—	+	—
	24 "	—	—	—	—	++	++
Cucumber virus 3	2 min.	+++	++	+	—	—	—
	2 hr.	++++	++++	++++	+++	++	—
	24 "	++++	++++	++++	++++	+++	++

In all tests the antiserum was used at a dilution of 1/50. 1 cc. of antiserum was added to tubes containing 1 cc. of antigen at given dilutions, and the tubes were immediately placed in a water-bath at 50° C.
+ signs indicate the degree of precipitation. — signs indicate that there is no precipitation.

reaction. CHESTER (1937*b*) has described a method whereby crude sap expressed through muslin is used as the antigen suspension in precipitation tests, and for some viruses he finds this to be more sensitive than using carefully clarified sap. This effect again may be a quantitative one. The viruses behaving in this manner may be present in the minimum quantities necessary for the precipitin test in crude sap, and clarification may reduce the virus content. What is probably a more important factor, however, is the presence of many unstable, easily-precipitable, substances in crude sap. The formation of a small specific precipitate, perhaps even one too small to see, will cause this unstable material to settle out, so making the reaction obvious.

CHESTER (1937*c*) has failed to obtain any specific precipitin reactions with extracts from plants infected with a large number of different viruses. At first sight this suggests that these viruses differ fundamentally from those such as potato virus "X" and tobacco mosaic virus which readily produce antisera and precipitate with them. But again the difference is more probably in the virus contents of plants. Those which readily react serologically are the more stable viruses occurring in high concentrations in infective sap. Of the viruses that have been found to give serologically active plant extracts, all grades of activity have been found, the exact grade being determined by the infectivity of expressed sap. Extracts of plants with tobacco mosaic will precipitate when diluted 1/10,000, of plants with potato "X" when diluted 1/1,000, whereas extracts of tobacco plants infected with potato virus "Y" rarely react when diluted more than 1/20, and sap containing the attenuated strain of Hyoscyamus virus 3 needs concentrating by ten times before it will give a precipitin reaction. It is probable that the application of the more sensitive techniques of complement fixation and virus-neutralisation to infective extracts not giving precipitin reactions would show that at least some contain specific antigens. With a number of animal viruses and bacteriophages these two phenomena have been demonstrated, although no precipitin reactions have been obtained.

Serological reactions as a quantitative test for viruses:— Because of their specificity the serological reactions can be used with certainty as a rapid method of identifying viruses. As the specific antigens in infected plants appear to be the viruses themselves, or at least the two are quantitatively related, the reactions can be adapted further to serve as quantitative tests for viruses. In Chapter 2 the local lesion method of quantitative working was described. If sufficient care is taken, this method gives fairly reliable results, but it is laborious and some days elapse after the inoculations are made before the lesions can be counted and the results determined. Using the precipitin test, equally accurate results can be obtained much more quickly and less laboriously. As the antisera remain constant over long periods, results obtained at different times with different preparations of a virus can be compared directly. With infectivity tests, because of variations in plants and in environmental conditions, this can never be done. Two methods can be used, the optimal precipitation or the precipita-

tion end point. The optimal precipitation point method is the quicker, but with the precipitation end point method smaller differences can be detected, especially when working with purified, heat-stable virus preparations. The two methods give the same results, *i.e.*, a preparation with four times the serological titre of another will also react optimally with serum at four times the concentration of the optimal value for the other.

The value of the precipitin test as a quantitative method was indicated by BEALE (1934), who first showed that the antigenic content of tobacco mosaic virus preparations was directly proportional to their infectivity. She used the precipitation end point method, the antigenic content being indicated by the greatest dilution at which a preparation will produce a visible precipitate when mixed with anti-serum. When two preparations were adjusted by dilution so that their antigen content was equal, they were equally infective. A few comparisons are given in Table 9. The relative antigen content was determined by titrating the virus preparations against a constant amount of antiserum, and the infectivity by inoculating to opposite halves of the same *N. glutinosu* leaves after the virus preparations had been diluted.

Table 9:
Tobacco mosaic virus. Comparison of infectivity and antigen content as indicated by precipitation end point

Precipitation end point	*) Dilution of inoculum	Mean number of lesions per half leaf
1/88	1/22	66
1/88	1/22	74
1/14	1/7	72
1/32	1/16	65
1/520	1/260	48
1/65	1/32.5	53
1/65	1/32.5	104
1/130	1/65	92

*) Inoculum diluted so that each pair has equal antigen content.

In measuring the relative antigen content of two virus preparations by the optimal precipitation point method, the antigen is used at a constant dilution and the concentration of the antiserum varied. To each of a series of tubes containing 1 cc. of antiserum at different dilutions is added 1 cc. of virus preparation. The tubes are immediately placed in the water-bath and the tube which first shows signs of precipitation is taken as containing the optimal amount of serum for that amount of virus. This amount of serum is directly proportional to the amount of antigen. If the same antigen preparation is tested undiluted and diluted 1 in 4, the undiluted sample reacts optimally with four times the concentration of serum reacting optimally with the diluted sample. Also, the relative antigen content of two similarly prepared suspensions as indicated by the concentration of serum with which they react optimally is directly proportional to their infectivity (BAWDEN 1935).

Although the serological tests can be used accurately in measuring the virus content of preparations, they do not always give the same results as infectivity tests. The two methods give the same results only when the preparations being compared have been treated similarly before testing and are equivalent in all respects other than virus content. The serological tests give a measure of the active antigen present, whereas the local lesion method indicates the amount of virus present capable of causing lesions. And, although there is no reason to doubt that the virus is the antigen, the ratio of serological activity to infectivity can be altered by a number of treatments. Substances such as formaldehyde and nitrous acid, or irradiation with X-rays and ultra-violet light, will cause complete loss of infectivity without in any way affecting the precipitating power of a virus preparation. Similarly, the addition of trypsin or some other proteins to virus preparations immediately reduces the number of lesions they produce, either by forming non-infective complexes or by reducing the susceptibility of the host, but has no effect on their reactions with antiserum.

Heating tobacco mosaic virus to 60-70° C, or precipitating with acid and salts can also affect the ratio between serological activity and infectivity. These treatments seem to increase the size of the virus particles by causing them to aggregate linearly. This aggregation reduces the infectivity of a given weight of virus and also affects serological measurements, for both optimal precipitation point and titre depend on the size and shape of the antigen particles. The serological method of measuring virus concentration can also give different results from the local lesion method if mixtures of virus strains are being tested. This effect is shown in Table 10, the results of an experiment in which two strains of potato virus "X" were tested separately and in mixtures of different proportions for their optimal precipitation points and their capacity to produce local lesions in *N. glutinosa* (BAWDEN 1935).

Each of the preparations tested contained the same amount of total virus, and this is indicated by the constancy of the optimal precipitation points (serum dilution 1/32), but not by the numbers of lesions, for only strain S causes local lesions in *N. glutinosa*. With viruses of the G type not giving easily-countable local lesions the serological method is especially valuable, but with all viruses it has many advantages over the infectivity method. However, for the reasons indicated, before the serological results can be accepted as a quantitative measure of active virus, they must be confirmed by infectivity tests.

Effect of participle-shape on serological reactions: — Bacteria with flagella give a different type of precipitate with antisera from non-motile individuals of the same species. When mixed with their antisera, the flagellate, or H-type, bacterial antigens are agglutinated rapidly, and form large clumps with a fluffy, open structure. By contrast, the somatic, or O-type, bacterial antigens are agglutinated more slowly and form smaller clumps which are dense and granular. Essentially similar differences are found between the form of precipi-

Table 10:

Potato virus "X". Comparison of mottle (G) and ringspot (S) strains, and mixtures of the two, for precipitating optima and production of local lesions

Antigen	Time	Serum dilution								Mean number of lesions
		1/8	1/16	1/32	1/64	1/128	1/256	1/512	0	
G	6 min.	—	—	+	—	—	—	—	—	0
	11 "	+	++	+++	++	+	+	—	—	
	20 "	+++	++++	++++	++++	+++	+++	++	—	
3/4 G 1/4 S	5 "	—	—	+	—	—	—	—	—	13
	9 "	+	++	+++	++	+	+	—	—	
	20 "	+++	++++	++++	++++	+++	+++	+	—	
1/2 G 1/2 S	7 "	—	—	+	—	—	—	—	—	20
	11 "	—	+	++	+	+	—	—	—	
	20 "	++	++++	++++	++++	+++	++	+	—	
1/4 G 3/4 S	6 "	—	—	+	—	—	—	—	—	63
	10 "	—	+	++	+	—	—	—	—	
	18 "	++	++++	++++	++++	+++	++	+	—	
S	8 "	—	—	+	—	—	—	—	—	120
	12 "	—	+	++	+	+	—	—	—	
	20 "	++	+++	++++	++++	+++	+++	+	—	

+ Signs indicate the degree of precipitation

tate produced by different viruses with their antisera. Strains of tobacco mosaic virus, cucumber viruses 3 and 4, potato viruses "X" and "Y" and Hyoscyamus virus 3 all give bulky flocculent precipitates which form rapidly. Tomato bushy stunt, tobacco necrosis and tobacco ringspot viruses, on the other hand, give granular precipitates which settle into small compact masses. These differences are illustrated in Fig. 22. The three left-hand tubes contain 1 cc. of bushy stunt virus antiserum at 1/50 and 0.1, 0.05 and 0.025 mgm. of bushy stunt virus in 1 cc. respectively. The three right-hand tubes contain 1 cc. of tobacco mosaic virus antiserum at 1/50 and 0.1, 0.05 and 0.025 mgm. of tobacco mosaic virus in 1 cc. respectively. After adding the diluted serum to the solutions of virus, the tubes were immediately placed in a water-bath at 50° C. A precipitate was at once obvious in the most concentrated solution of tobacco mosaic virus, and within two minutes there was a large precipitate in this and precipitation was also obvious in the more dilute solutions. The first signs of precipitation were not apparent in the most concentrated solution of bushy stunt virus until the tubes had been in the bath for six minutes, and it was half an hour before a precipitate settled out of the most dilute solution. After twelve hours in the water-bath, the tubes were left undisturbed for a further twelve hours at room temperature when they were photographed.

The character of the specific precipitate is almost certainly determined by the shape of the virus particle. Tobacco mosaic has

rod-shaped particles and all the other viruses giving nebulous precipitates also have particles that are readily orientated by streaming. The particles of bushy stunt, tobacco necrosis and tobacco ringspot viruses, however, are not orientated by streaming and all the available evidence suggests that they are spherical or nearly so. Structurally, therefore, the viruses giving H-type precipitates resemble flagella and those giving O-type precipitates resemble the bodies of bacteria, and it is to be expected that spheres would pack more tightly and give a more compact precipitate than rods.

Antisera to somatic type bacterial antigens are known to differ in several ways from those to flagellar antigens; they lose their ability to cause agglutination more easily on heating or ageing and they also lose this ability when treated with formalin. In all these properties, the antisera to bushy stunt and tobacco mosaic viruses also differ. After heating for 10 minutes at 75° C, bushy stunt virus antisera cease to precipitate the virus, whereas tobacco mosaic virus antisera still cause precipitation when heated to over 80° C. Similarly, after treatment with formalin, bushy stunt virus antisera do not cause precipitation, although tobacco mosaic virus antisera do, and antisera to bushy



FIG. 22. — Precipitates of tomato bushy stunt and tobacco mosaic viruses with their homologous antisera. Three left-hand tubes, 0.1, 0.05 and 0.025 mg. of bushy stunt virus; three right-hand tubes, same weights of tobacco mosaic virus; central tube saline control. Note dense, granular precipitate of bushy stunt and bulky, flocculent precipitate of tobacco mosaic virus. (BAWDEN, F. C. and PRIE, N. W., 1938, *Brit. J. exp. Path.* 19, 251).

stunt virus may cease to cause precipitation after storage for one or two years at 1° C whereas those to tobacco mosaic virus still precipitate after 10 years.

The effects of heating on antisera to the two kinds of antigen have been studied in detail by KLECZKOWSKI (1941*a* and *b*). It was widely believed that the antibodies to O- and H-type antigens differed in their resistance to heating, but KLECZKOWSKI has disproved this by isolating globulin fractions of the two antisera and heating them separately, when he found that both behave in the same way and continue to cause precipitation until heated for 10 minutes at 90° C. When whole sera are heated at temperatures between 75° C and 90° C, antibodies apparently combine with other proteins undergoing heat denaturation. The behaviour of such complexes in precipitation tests depends on the protein with which the antibody has combined and on the antigen. Complexes with euglobulin behave much like unchanged antibody, whereas complexes with albumin, which predominate when whole sera are heated, can combine with their antigens but not precipitate them. Antisera to H-type antigens appear to be more heat-stable because these antigens are more readily flocculated by mixtures of normal antibody and antibody-albumin complexes than are O-type antigens.

Similar differences are obtained when viruses of different shapes are heated with albumin, for the viruses as well as their antibodies can form complexes with other proteins (BAWDEN and KLECZKOWSKI 1941, 1942). Complexes of tobacco mosaic virus and albumin precipitate with antiserum whereas complexes of bushy stunt virus and albumin do not, although they still combine with antibodies for they inhibit the precipitation of unchanged bushy stunt virus. These non-precipitating complexes of bushy stunt virus are still antigenic, for they produce antisera indistinguishable from ordinary virus antisera when injected into rabbits, and they also fix complement with virus antisera. The difference in the serological behaviour of the two viruses after heating with albumin probably lies in the fact that tobacco mosaic virus is much more easily rendered insoluble by combination with antibody than is bushy stunt virus; its floccules not only separate quicker, but they are produced with less antibody and over a wider range of antigen/antibody ratios. It is probable that incorporating relatively small amounts of soluble protein in the particles formed by the union of antigens and their antibodies is sufficient to keep them in solution if the antigens are O-type but not if they are H-type.

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METHODS OF PURIFICATION

The fact that tobacco mosaic virus could be precipitated by protein precipitants and resuspended without losing its infectivity was shown by the early work of MULVANIA (1926) and VINSON and PETRE (1929; 1931). Similarly, MACCLEMENT (1934) found that potato virus "X" was amenable to purification methods used for the isolation of some enzymes, and obtained preparations free from many normal plant constituents by precipitating infective sap at different pH values. This early work showed the possibilities of isolating the viruses in a pure state, but it produced little direct evidence on the chemical nature of viruses because no attempts seem to have been made to concentrate the viruses and obtain them in sufficient quantities for detailed chemical examination. STANLEY (1935), working with tobacco mosaic virus and using a modification of NORTHROP's methods for the isolation of proteolytic enzymes, first showed that a plant virus might be handled *in vitro* as a protein and obtained in bulk. This provided the stimulus for the later work, which has resulted in the separation of several viruses in apparently pure states.

The same general methods have been used for all the viruses isolated, but they need to be varied with individual viruses as these precipitate in different conditions and vary in stability. Sap from diseased plants is subjected to a variety of treatments, which precipitate either the viruses or normal plant constituents, or which destroy these constituents but not the viruses. Purification of all the viruses is facilitated by choosing suitable starting material. Young plants are preferred, as old ones contain much pigmented material that is difficult to remove without drastic methods of fractionation. Purification is often easier in the winter than in the summer, for plants growing during the winter contain small amounts of constituents that interfere with purification. With some diseases, the virus content of infective sap is widely different at different times after infection, and so the time of extracting the leaves is also important. In general, for optimal conditions, that is for greatest yield and ease of isolation, young plants should be inoculated and their sap extracted when they are showing most definite symptoms.

Although there is every reason to believe that the isolated proteins are the viruses themselves, the exact relationship between the purified preparations and the viruses as they are produced in infected cells is by no means clear. The ideal purified virus preparation would contain particles all identical in size, constitution and infectivity, but there is no evidence that this ideal has been achieved. Three ways in which deviations from this ideal can arise are already known. An obvious one is that the preparations may contain host constituents inessential for infectivity. The early preparations of tobacco

mosaic virus were of this type, but the detection of such contaminants should present few serious problems, and it is probable that several viruses have been made free from them. The fractionations necessary to get rid of such contaminants, however, may modify the virus particles so that although the final product contains only virus material, the particles have different sizes or activities. When the modification is slight the detection of such mixed products may present considerable difficulties. All preparations of tobacco mosaic virus probably fall into this category, for most treatments seem to cause the particles of this virus to aggregate. The first preparations of tomato bushy stunt virus, in spite of their apparent physical homogeneity, were mixtures of infective and non-infective virus particles, for the method used involved heating the sap and this is now known to cause considerable inactivation. Even if the preparations contain nothing except fully active virus particles, these may be of different kinds. Such a deviation from ideal homogeneity may be independent of the technique of isolation, for such mixtures occur within the plant. It is probable that all preparations of tobacco mosaic virus are of this type, for mutation is so frequent that even cultures derived from single local lesions are mixtures of virus strains. Similarly, preparations of tobacco necrosis viruses are often mixtures of particles differing both serologically and in their ability to cause infection.

Until more sensitive tests for activity are developed, so that individual particles can be shown to be infective, proof that any virus preparation is homogeneous is not likely to be possible. The methods described below for the purification of some viruses give products which appear homogeneous in all their physical properties and which weight for weight are as infective as virus in infective saps. This is the only test now available for full infectivity, but it is not necessarily a valid one and more specific methods of isolation may in the future give more active preparations. At the present time, to ensure removing all host contaminants, it is necessary to expose the viruses to fractionation treatments that might affect the homogeneity of the preparation.

Tobacco mosaic virus: — The method first described by STANLEY (1935, 1936*a*) for the purification of tobacco mosaic virus consisted essentially of repeated precipitations with 40% saturated ammonium sulphate solution, the precipitated virus being adsorbed on to celite (a diatomaceous filter-aid) and then eluted in water. Precipitation with lead subacetate at pH 8.8, or treatment with an aqueous suspension of calcium oxide (1936*b*) at pH 8, was used to remove most of the pigmented material. However, these treatments reduced the yield of virus and later STANLEY (1937*a*) states that the virus can be isolated from young plants by the following method.

About three weeks after infection, young plants are cut down and frozen. They are minced in a meat mincer while frozen, and after thawing the sap is expressed through muslin. The sap is adjusted to pH 7.2 by the addition of NaOH. As all the virus is not obtained in the first extract, the alkaline juice is added to the minced leaves,

thoroughly mixed, and then again expressed. Next the extract is clarified by filtration through a layer of celite on a Buchner funnel. To the clarified sap is added 30% by weight of ammonium sulphate. The resulting precipitate is collected by filtration through celite and the coloured filtrate discarded. The celite is taken up in water at pH 7, and again filtered. In these conditions the virus dissolves and is found in the filtrate. The precipitations with ammonium sulphate are now repeated, using less than in the first precipitation, until the filtrate from the ammonium sulphate precipitation is colourless. The precipitate is then dissolved in water and brought to about pH 4.5 with acid. This causes the separation of a precipitate containing the virus which is collected by filtration through a thin layer of celite. The celite filter-cake is suspended in water, brought to pH 7 and the celite removed by filtration. The addition of acid or ammonium sulphate to the opalescent filtrate causes the separation of a precipitate with a characteristic satin-like sheen, composed of microscopically visible needle-shaped bodies (Fig. 23).

STANLEY (1937*b*) regarded these needles as true crystals and as the activity of his preparations was not affected by repeated precipitations with acid and ammonium sulphate (or "recrystallisations") he concluded that they were pure. However, CHESTER (1936) showed by the sensitive anaphylactic test that the preparations contained normal plant proteins in addition to tobacco mosaic virus. BAWDEN and PIRIE (1937*a, b, c*) also showed that precipitations with acid and salts were usually insufficient to give homogeneous preparations. They found that the appearance of the precipitated needles is not significantly affected by the presence of comparatively large quantities of certain impurities, and that the apparent uniformity of the precipitated material cannot be taken as evidence of purity. Another property of the virus preparations is much more sensitive to the presence of impurities. This is their ability to form liquid crystalline solutions. BAWDEN and PIRIE found that highly purified preparations of tobacco mosaic virus, if more concentrated than about 1.6%, settle into two layers on standing undisturbed. The lower layer is the more concentrated and is spontaneously birefringent, *i.e.*, it is liquid crystalline and visible between crossed Nicol prisms. The upper layer is not birefringent when stationary, but on gentle agitation shows the phenomenon of anisotropy of flow strongly. These phenomena are described in greater detail later. They are mentioned here because of their application in the purification method. Impure preparations show anisotropy of flow less strongly; they either do not form liquid crystalline solutions or do so only when much more concentrated than highly purified preparation. As no materials have been found in healthy plants which show anisotropy of flow, examination of the fluids during the course of preparation by shaking between crossed Nicol prisms is the most convenient and rapid method of testing for the presence of the many viruses showing the phenomenon.

The following method of preparation gives colourless products that readily form dilute liquid crystalline solutions. Infective sap is frozen, thawed and centrifuged. The supernatant fluid is adjusted to pH 3.3 by the addition of N/10 HCl, when a precipitate separates,

which is centrifuged off. The precipitate is suspended in water and dilute NaOH added to bring the pH to 7, when the fluid is again centrifuged until clear. The darkly coloured precipitate is discarded, and the virus again precipitated by the addition of one-third of a volume of saturated ammonium sulphate solution. After centrifuging, the coloured supernatant fluid is discarded, the precipitate is taken up in water and the precipitations with one-quarter saturated ammonium sulphate solution repeated until the supernatant fluid is colourless.

The product from the final precipitation with ammonium sulphate is dissolved in water, using about 50 cc. per litre of original sap, and N/10 HCl added to bring the pH to 3.3. The virus-containing precipitate is again centrifuged off, and is washed by being suspended

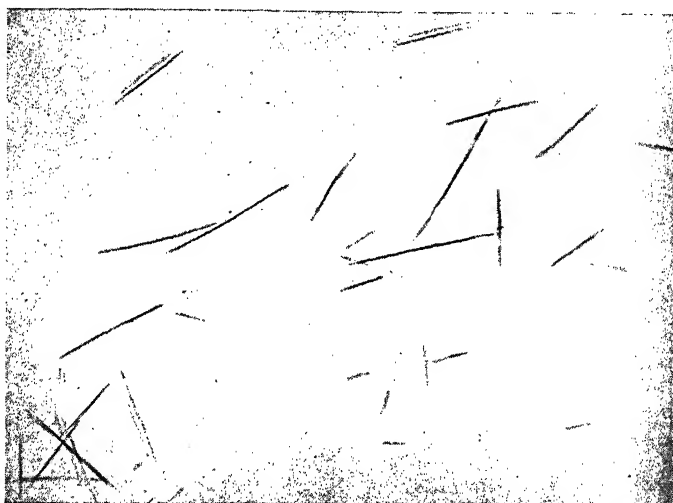


FIG. 23. — Needle-shaped paracrystals of tobacco mosaic virus produced by precipitation with acid and ammonium sulphate. $\times 675$. (STANLEY, W. M., 1937, Amer. J. Bot. 24, 59).

in water and centrifuged. The virus is insoluble at pH 3.3 only in the presence of salts, and when the washings have reduced the salt content sufficiently the virus goes into solution. The well-washed precipitate is then taken up in 50 cc. of water at pH 3.3 and centrifuged, when a small amount of impurity is usually deposited. The virus can now be precipitated from the supernatant fluid either by the addition of some salt or by raising the pH to 4.2 by the addition of dilute NaOH. The suspension is centrifuged, the precipitate dissolved in sufficient NaOH to give a final solution at pH 7, and then again centrifuged at 3,000 r.p.m. until clear.

Solutions prepared in this manner are opalescent and sometimes almost colourless. If more concentrated than 4% they may settle into two liquid layers. More often they are coloured, and do not settle into layers. Further precipitations with acid and ammonium sulphate have little or no effect in improving such preparations, although the fact that they can be fractionated by other treatments

shows that they still contain contaminants. The method most generally useful for further purification is incubation with trypsin (BAWDEN and others 1936; BAWDEN and PIRIE 1937*a*). Solutions containing from 0.5 to 2% of solids and 0.2% of commercial trypsin, with a drop of chloroform added as a disinfectant, are incubated at 37° C and at pH 7.5 to 8 for about 24 hours. After this treatment, the preparation readily becomes colourless with two or three further precipitations with acid or ammonium sulphate, and neutral solutions at about 2% concentration are liquid crystalline. The precipitations seem to free the preparations completely from trypsin. No differences in activity or other properties have been noticed between virus purified with trypsin and that purified by other methods, but the possibility cannot be excluded that the final product is a virus-enzyme complex.

As an alternative to incubation with trypsin, the partially purified preparations may be further fractionated by high speed centrifugation. The virus-particles are larger than any other of the components of the partially purified preparations and so sediment more readily when exposed to intense centrifugal fields. Neutral, salt-free solutions of tobacco mosaic virus are unaffected by long periods of centrifuging at 3,500 r.p.m.; to obtain a sediment at this speed it is necessary to add sufficient acid or salt to force the virus out of solution. But if the centrifugal field is increased, the forces keeping the particles in solution are overcome, and the virus can be sedimented. Coloured preparations made by the precipitation methods described, which do not give a liquid crystalline layer unless more concentrated than 5%, deposit a birefringent layer when centrifuged at high speed. When a 2% neutral solution is centrifuged for 2 hours in a centrifugal field of 16,000 times gravity, about half the total virus is sedimented in the form of a highly birefringent jelly. When this jelly is dissolved in water it gives solutions that layer and are liquid crystalline at a concentration of about 2%.

The layering phenomenon itself can be adapted for the further fractionation of contaminated preparations, for the impurities tend to be concentrated in the upper layer. If, from a preparation which is layering only when concentrated, the bottom layer is separated from the upper, diluted with water and allowed to stand for some time, this diluted solution will again settle into layers. This new lower layer can again be separated and diluted, and the process repeated until a product can be obtained forming a liquid crystalline layer at a concentration as low as 1.6%. However, only a small part of a virus preparation can be obtained in a highly purified form by this method. Another disadvantage is its slowness, for after separating the layers and diluting the bottom one, sufficient time must elapse for the solutions to layer again before the process can be continued. Processes that lead to a decrease in the concentration at which preparations become liquid crystalline, also lead to significant increases in serological titre. This suggests increased purity, but as the processes select the longer particles the interpretation of this fact is not simple.

A number of different strains of tobacco mosaic virus have been

successfully purified by these methods. None of these show any significant differences in their behaviour. With slight variations the methods can also be adapted for the purification of cucumber viruses 3 and 4 (BAWDEN and PIRIE 1937*b*). These viruses give liquid crystalline solutions and show anisotropy of flow; they also precipitate in the form of needles, microscopically resembling those of the tobacco mosaic viruses, when ammonium sulphate or acid is added. But less acid is needed to cause the precipitation, the optimal precipitation point with acid being about pH 4.8 instead of pH 3.3. This fact may in part explain the different host ranges of the two groups of virus, for whereas the pH of expressed cucumber sap is between pH 7 and 8 that of expressed tobacco sap is between pH 5 and 6.

Potato viruses "X" and "Y":— Solutions of potato virus "X" resemble those of tobacco mosaic virus in that they show anisotropy of flow and form liquid crystalline layers, but the precipitates formed by salt or acid are amorphous. The separation of solution into layers is even more sensitive to the presence of impurities than with tobacco mosaic viruses, and occurs only with highly purified preparations. Hence, although the phenomenon is again useful as an indicator of purity, it has no value as a method of fractionation. Both potato viruses "X" and "Y" adsorb impurities much more readily than do tobacco mosaic viruses, and their purification is more difficult.

Sap from tobacco plants infected with potato virus "X" is frozen, and thawed and anhydrous disodium hydrogen phosphate added at the rate of 15 gms. per litre. After centrifuging, the brown supernatant fluid is one-quarter saturated with ammonium sulphate (185 gms. per litre) or brought to pH 4.5 by the addition of $N/10$ HCl. Both treatments produce a fawn-coloured, flocculent precipitate containing all the virus. This is centrifuged off, suspended in a volume of water equal to one-tenth of the original sap, neutralised with NaOH, and centrifuged until clear. The supernatant fluid if shaken between crossed Nicol prisms (or polaroid plates) now shows definite anisotropy of flow visible to the naked eye.

The precipitations with ammonium sulphate are repeated until the supernatant fluids are no longer brown, a few drops of dilute NaOH being added each time to keep the fluids neutral. The precipitate is dissolved in water and centrifuged until clear, when the supernatant fluid is poured off and adjusted to pH 4.5 by the addition of acetic acid. The resulting precipitate usually contains the whole of the virus, but if the salt content of the preparation is small the supernatant fluid may still show definite anisotropy of flow. The virus remaining in solution at pH 4.5 can usually be precipitated by adjusting the pH to either 4 or 5. The product is freed from ammonium sulphate by washing at pH 4.5 with water, but as the salt content is reduced a larger proportion of the virus becomes soluble at pH 4.5. This effect can be counteracted in part by freezing the acid precipitate solid, and then thawing before centrifuging. After this treatment most of the virus can be sedimented by centrifuging at 3,000 r.p.m., and as it also packs much more tightly than the unfrozen precipitates, it can be taken up in less water to give a more concentrated solution.

Although preparations at this stage show the phenomenon of anisotropy of flow strongly, they are always brown and never liquid crystalline. Further precipitations with acids or salts effect no appreciable improvement, but the preparations can be further purified by incubation with trypsin or by high speed centrifugation. As virus "X" is hydrolysed by trypsin, the incubation must be carried out with greater caution than with tobacco mosaic virus. Preparations containing about 0.4% of solids are incubated with 0.05% commercial trypsin for 90 minutes at pH 7.5 and 38° C. This treatment destroys about a third of the virus, but contaminants are more susceptible to tryptic digestion, so that the incubated preparations usually become colourless and form liquid crystalline solutions after a few further precipitations with ammonium sulphate and acid. Potato virus "X" sediments in a high speed centrifuge more slowly than tobacco mosaic virus; impurities also tend to adhere to it more firmly, so that repeated centrifugation is necessary for its purification. When the partially purified preparations containing from 1 to 2% of solids are centrifuged for 3 hours in a centrifugal field of 16,000 times gravity, from a fifth to a third of the virus sediments. The pellets formed are turbid and birefringent, and have a solid content of from 15 to 20%. They dissolve in water to give opalescent, faintly coloured solutions from which brown material, rich in carbohydrate, can be removed by centrifuging at 3,000 r.p.m. The supernatant fluid is again centrifuged for 3 hours at high speed. The birefringent pellets from the second centrifugation still contain small amounts of insoluble materials, but they are now colourless. The insoluble material is removed by suspending the pellets in water and centrifuging at low speed. The virus remaining in the supernatant fluids after the high speed centrifugation can be recovered either by the use of trypsin, or by further high speed centrifugation after concentration by precipitation with acids and ammonium sulphate.

Potato virus "Y" occurs in sap in much smaller quantities than either tobacco mosaic virus or potato virus "X". As it is also very labile, adsorbs impurities readily and in its general properties resembles some of the normal plant proteins, its purification is considerably more difficult. Infected tobacco plants also contain a lipid-containing substance and this has only been separated from the virus by repeated differential centrifugation (BAWDEN and PRIE 1939).

Disodium hydrogen phosphate is added to infective sap until a copious precipitate is produced. This is centrifuged off, and 300 gms. of ammonium sulphate is added to each litre of the supernatant fluid, together with some ammonium hydroxide to keep the mixture neutral. This is necessary because much of the virus is lost if the pH falls sufficiently to coagulate the plant protein. After centrifuging thoroughly, the precipitate is suspended in a measured volume of water (equal to about one-fifth of the volume of original sap) and centrifuged. The supernatant fluid is one-fifth saturated with ammonium sulphate, centrifuged after one hour and the precipitate discarded. The virus is now precipitated from the supernatant fluid by bringing the content of ammonium sulphate up to two-fifths of saturation.

The precipitate now is a soft brown mass which will not pack tightly in the centrifuge. It is suspended in one-fifth of its volume of phosphate buffer at pH 8 and dialysed for a few hours against tap-water. After dialysis the solution is incubated for 6 hours with trypsin, and then again one-fifth saturated with ammonium sulphate. The precipitate formed is discarded, and the supernatant fluid one-third saturated with ammonium sulphate, when it is centrifuged for one hour to pack the precipitate tightly. This precipitate is suspended in a small volume of water, the pH adjusted to 7.5, and any insoluble material centrifuged off.

The final supernatant fluid is then further fractionated by high speed centrifugation. Most of the virus sediments in 3 to 4 hours at 16,000 r.p.m. to form opaque pellets. These are suspended and centrifuged for an hour at 10,000 r.p.m., which deposits some contaminants but little virus. The process of differential centrifugation, that is 3 to 4 hours at 16,000 r.p.m. followed by shorter periods at 10,000 r.p.m. on the resuspended pellets, is continued until the pellets are all homogenous and birefringent. The product obtained is a nucleoprotein which precipitates with salt and acid in much the same way as potato virus "X". A good deal of the virus is lost on the precipitates discarded at various stages in the preparation and the final product contains much virus that is serologically active but not infective.

Tomato bushy stunt and tobacco necrosis viruses:—The only other viruses yet purified by precipitation methods are those causing tomato bushy stunt and tobacco necrosis (BAWDEN and PRIE 1938b, 1942; PRIE and others 1938). These viruses resemble one another in many ways, but they precipitate in different conditions from those whose purification has been described above. They are soluble over the whole pH range in which they are stable and in the presence of salt they are more soluble at $0^{\circ}C$ than at $20^{\circ}C$. These viruses can be rendered non-infective by heat, ageing and alkali, while still retaining their specific serological and physical properties, so that apparently homogeneous end-products are often mixtures of active and inactive virus particles. For good yields, young plants should be inoculated, preferably in the winter, and for tobacco necrosis viruses, which give only local lesions, the inoculum should be a concentrated one.

Whole tomato plants suffering from bushy stunt are minced and their sap expressed. For each 100 cc. of sap, 30 cc. of a 4% solution of anhydrous disodium hydrogen phosphate is added to the leaf residue, which is again minced and the sap expressed. The two extracts are mixed and centrifuged, and ammonium sulphate is added to the supernatant fluid at the rate of 280 gms. per litre. After standing, the precipitate is centrifuged off and resuspended in a volume of water equal to one-tenth of the original sap. This fluid is centrifuged and the precipitate extracted twice more with smaller volumes of water. The three extracts are mixed, brought to pH 4 with acetic acid and spun. Ammonium sulphate is now added to the supernatant fluid until there is turbidity not dissipated by stir-

ring. A precipitate containing most of the virus separates within a few hours at room temperature and is centrifuged off. It is extracted two or three times with dilute acetate buffer so that the combined extracts equal about one-hundredth of the volume of the original sap. Saturated ammonium sulphate solution is now added drop by drop at room temperature until the fluid becomes perma-

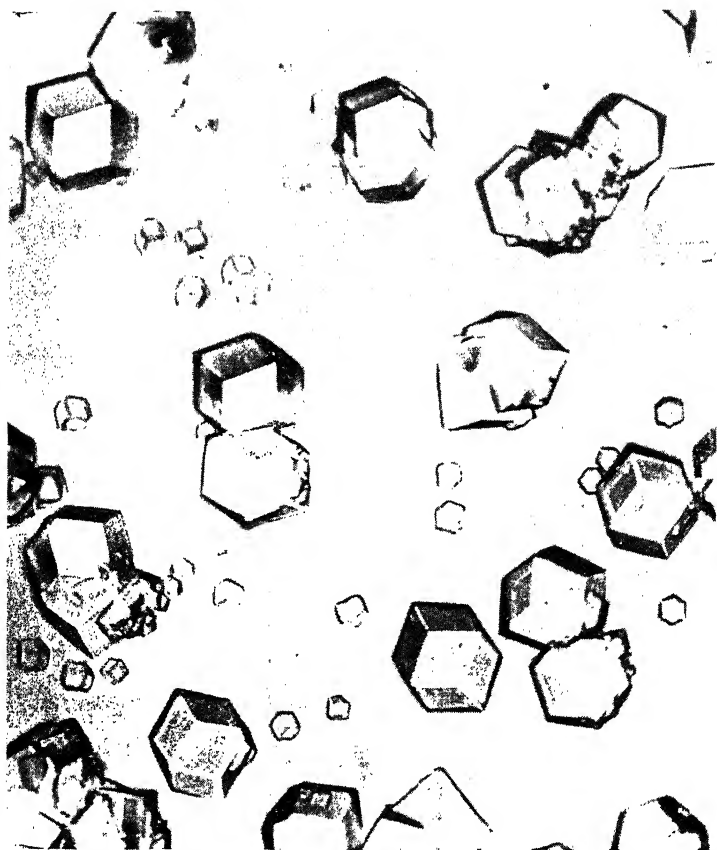


FIG. 24. — Dodecahedral crystals of tomato bushy stunt virus produced by slow precipitation with ammonium sulphate in the cold. The crystals were photographed on the wall of a vessel in which the same preparation was twice crystallised. The larger crystals were produced by the first crystallisation and the smaller by the second. $\times 250$. (BAWDEN, F. C. and PIRIE, N. W., 1938, *Brit. J. exp. Path.* 19, 251.).

nently opalescent, when it is cooled to 0°C and becomes clear. After a few hours at 0°C any insoluble material is removed by centrifugation at 0°C . This is most conveniently done by running the centrifuge inside a refrigerator, but if this is impossible it can be done by placing small tubes with the virus solution inside larger centrifuge tubes containing water and crushed ice. After 15 minutes at 2,000 r.p.m. the solution will still be at 0°C . The precipitate is discarded and the supernatant fluid kept at 0°C . Crystallisation should start within a few hours, but if it does not a little more ammonium sulphate solution should be added. The process of crystallisation is

allowed to proceed for 48 hours, when the fluids are centrifuged for a few minutes in the cold. The crystals dissolve more slowly in water than the amorphous material or than any soluble contaminants. They are therefore suspended in about three times their volume of water and centrifuged off again immediately. After this washing, they are again suspended in water and left for an hour with frequent stirring to dissolve, when any contaminating insoluble material is removed by a further period of centrifugation.

Preparations of the virus at this stage are faintly opalescent and almost colourless. If the careful precipitation with ammonium sulphate at 0°C is repeated, they crystallise completely (Fig. 24), and

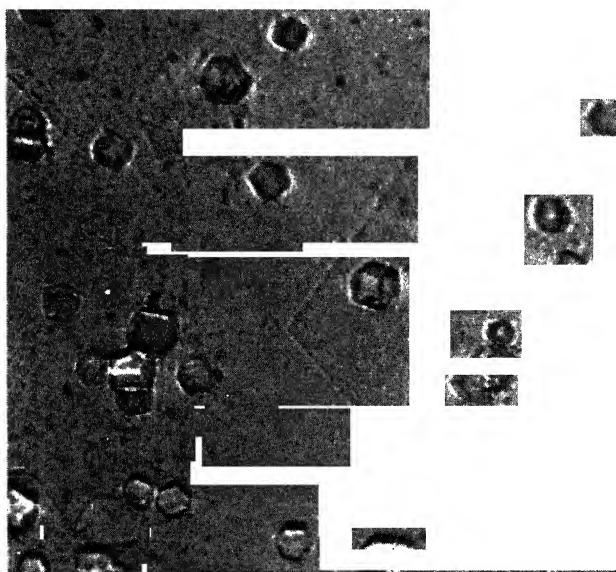


FIG. 25. — Crystals from a mixed culture of tobacco necrosis viruses obtained from the roots of a naturally infected tobacco plant. Flat plates and dodecahedra are found together. $\times 70$.

the crystals dissolve to give quite colourless and apparently homogeneous solutions. As bushy stunt virus is soluble at its isoelectric point, the preparations cannot be freed from salt by precipitation with acid and repeated washing of the precipitate with water. The solutions of crystals are therefore enclosed in cellophane sacs and thoroughly dialysed against distilled water. If dialysis is carried out at $\text{pH } 3$ instead of $\text{pH } 7$ a further small fractionation is sometimes obtained, for a colourless precipitate, consisting of a mixture of carbohydrate and denatured virus, may separate and can be centrifuged off when dialysis is complete.

Not all the virus is obtained from the coloured preparations by the slow crystallisation in dilute ammonium sulphate solution at 0°C . When the crystallisation mother liquids are warmed up to room temperature, they deposit an amorphous precipitate rich in virus. This can be recovered, after concentration by further precipitation and

resolution in a smaller volume of water, by repeating the crystallisation process.

The different viruses causing tobacco necrosis vary in their resistance to the processes of purification, some being rendered non-infective by treatments that apparently have no effect on others. No method of purification by precipitation methods has been found that is uniformly successful. For the unstable viruses, the method described above for bushy stunt virus gives least inactivation, whereas the following method gives the best results with others, although it sometimes fails to give good yields (PIRIE and others 1938; BAWDEN and PIRIE 1942).

Minced leaves are frozen, thawed and the sap expressed. The

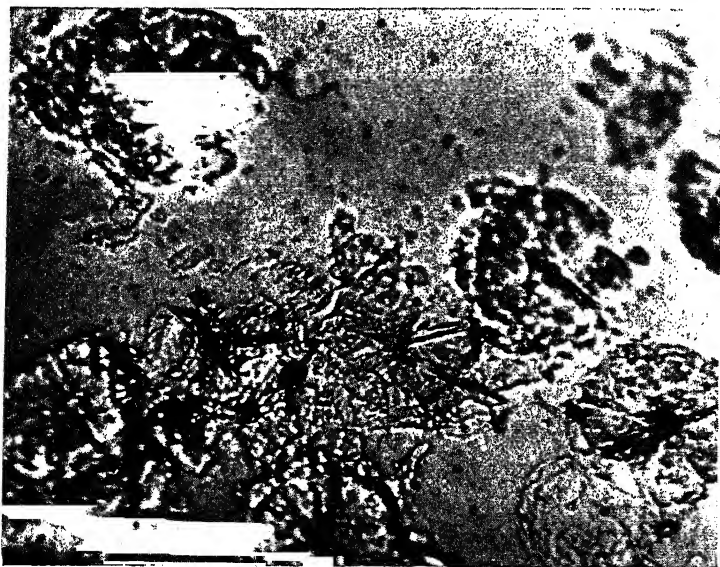


FIG. 26.— Crystals of a tobacco necrosis virus in the form of round laminae. An alternative crystal form of this virus is shown in Fig. 27. $\times 200$

leaf residue is extracted with a volume of 4% solution of disodium hydrogen phosphate equal to one quarter of the sap, and the sap and extract are mixed. Half a volume of 90% alcohol is added to the mixture and the precipitate discarded. The pH of the supernatant fluid is brought to between 4 and 4.5 by the addition of hydrochloric acid, and the precipitated material is discarded. Two volumes of alcohol are thoroughly mixed with the supernatant fluid, and, after lying for an hour, as much as possible of the liquid is decanted. The precipitate contains the virus and is packed tightly by centrifuging, after which it is extracted with a 3% solution of ammonium sulphate in 0.5% acetic acid. This converts the calcium salts, of which the precipitate is largely composed, into hydrated calcium sulphate, and allows the virus to go into solution. Three extracts are usually sufficient, provided their combined volume is about one-tenth that of the original sap. Half a volume of saturated ammonium sulphate

is added to the extracts and the mixture is allowed to stand for at least 5 hours before centrifuging for 45 minutes at 3,500 r.p.m. With some preparations, the ammonium sulphate will produce definite flocules, whereas with others the virus content is too small for anything more than an increased opalescence. The precipitate is clear and colourless, and is extracted two or three times with water, using a total volume amounting to about one-hundredth of the sap.

Saturated ammonium sulphate solution is now added at room temperature until a definite opalescence is produced, when the fluid is

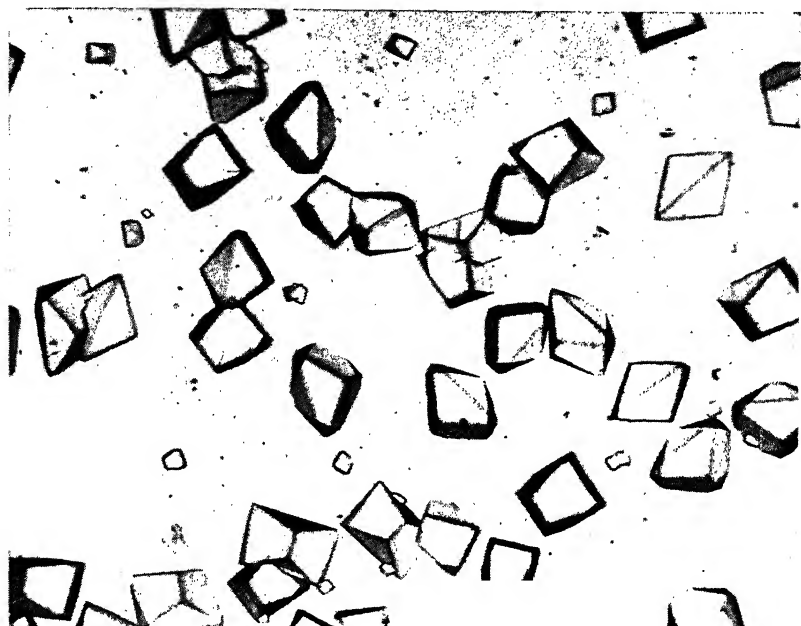


FIG. 27. — Crystals in the form of bi-pyramids formed by the same virus as shown in Fig. 26. A further form of crystallisation, dodecahedra, is shown in Fig. 25. The bi-pyramids are birefringent. $\times 70$.

cooled to 0°C and treated as already described for bushy stunt virus preparations.

The result of storing at 0°C in ammonium sulphate solution varies with different sources of the virus. Many crystallise and sometimes give mixtures of crystals. For example, Fig. 25 is a photograph of a preparation that gave both thin, lozenge-shaped crystals and dodecahedra. This culture contained two serologically unrelated viruses, which were separated by isolating single local lesions and multiplying the virus from each separately. Most cultures from single local lesions behave uniformly, although one has been found to give different crystal forms in successive preparations. This has crystallised as approximately circular laminae (Fig. 26), rhombic dodecahedra (Fig. 25) and as birefringent bi-pyramids (Fig. 27). The causes determining the crystal form are unknown; if one sample of infected sap is divided into two and the halves purified separately, one sometimes crystallises as dodecahedra and the other as bi-pyramids. Simi-

larly, if dodecahedra are dissolved and recrystallised, bipyramids or circular laminae are often formed. Attempts to influence the crystal form by "seeding" the preparation set to crystallise have failed. Other tobacco necrosis viruses have crystallised as birefringent hexagonal prisms, sometimes with pointed ends (Fig. 16) and sometimes with points replaced by facets (Fig. 35), while still others have failed to give any recognisable crystals. When placed in the cold in ammonium sulphate solution, preparations of all the tobacco necrosis viruses usually develop a sheen after a few hours. With some this is the only effect, however long they remain, but with others crystallisation then begins. The time for this again varies with individual viruses and virus strains; with the one forming lozenge-shaped plates, crystallisation may be complete in a day or two, but with the one forming hexagonal prisms a fortnight or more may be necessary.

Purification by high-speed centrifugation:—In the methods of purification already described high speed centrifugation has been mentioned as a method of fractionating partially purified preparations, but it is also widely in use for isolating viruses direct from infective sap. This has been made possible by the development of air-driven ultra-centrifuges capable of holding about 100 cc. of fluid and giving centrifugal fields of 60,000 times gravity or more. The first work of this type was done by WYCKOFF and COREY (1936), who found that tobacco mosaic virus sediments from clarified sap to form a birefringent pellet. The method has usually been applied to sap from frozen leaves and may partly owe its success to this fact. Infected leaves are frozen overnight at -14°C , minced and the sap extracted. After clarification by filtration or low speed centrifugation, the sap is centrifuged for from $1\frac{1}{2}$ to 3 hours in a field of from 40,000 to 90,000 times gravity, the time and the centrifugal field depending on the ease with which the particular virus sediments.

The supernatant fluids are discarded and the pellets are suspended in water or phosphate buffer. The suspension is clarified by low speed centrifugation and then again subjected to a further period of ultra-centrifugation. This process of alternate centrifugations at high and low speeds is repeated three or four times, until no protein can be detected in the supernatant fluid from the high speed centrifugation and the sedimented pellet dissolves completely.

These machines are costly and have limited capacities, so that the production of viruses in large quantities is difficult except for those like tobacco mosaic virus and potato virus "X" which occur in sap in fairly high concentrations. Using only high speed centrifugation these two viruses have been sufficiently purified to form liquid crystalline solutions (LAUFFER and STANLEY 1938; LORING 1938*b*), which suggests that the preparations contain little material other than virus. This suggestion is supported by the similar analytical figures found for the viruses purified by this and by other methods. High speed centrifugation is a less drastic method, and causes less change in the activity and filterability of these viruses, than precipitation with acids, salts or alcohol. BAWDEN and PIRIE (1937*a*, 1938*a*) found that these viruses were less infective and fil-

tered less readily after purification and they suggested that precipitation caused the virus particles to aggregate linearly. LORING, LAUFFER and STANLEY (1938) confirmed this with tobacco mosaic virus, but claim that such aggregation is avoided if high speed centrifugation is used as the sole method of purification. LORING (1938b) also found that preparations of potato virus "X" made by this method are more active and more consistent in their infectivity than those made by precipitation methods, and he suggests that acid and strong salt solutions produce slight chemical changes in this virus which are responsible for loss of infectivity. Great care, however, seems to be needed in the handling of preparations to prevent aggregation. WYCKOFF (1937) found that homogenous preparations of tobacco mosaic virus made by centrifugation became inhomogenous if merely exposed to phosphate buffer at pH 7, and even when the pellets were dissolved in water LORING, LAUFFER and STANLEY (1938) detected changes after the virus had been sedimented four times. FRAMPTON'S (1942) measurements of the lengths of tobacco mosaic virus particles also show that many of them in preparations made by centrifugation were aggregates. Similarly, there was considerable molecular heterogeneity in the preparations of potato virus "X" made by LORING and WYCKOFF by ultracentrifugation. Such aggregation may be a necessary corollary of a high degree of purity, for BERNAL and FANKUCHEN (1941) suggest that the tendency of the particles to form linear aggregates is increased by the removal of contaminating substances from their ends. Thus it is possible that the preparations with particles behaving like those in infective sap were merely those in which purification was not carried sufficiently far to remove such contaminants.

With viruses other than tobacco mosaic and potato virus "X", there is little evidence that ultracentrifugation on its own is sufficient for the isolation of pure viruses. The early claims that the materials sedimented from infective sap were the viruses, were largely based on the statement of WYCKOFF, BISCÆ and STANLEY (1937) that sap from healthy tobacco plants contains no material with a molecular weight greater than 30,000. However, this is not so. PRICE and WYCKOFF (1939) have found substances giving a sedimentation constant of 74×10^{-13} in expressed sap from both tobacco and *N. glutinosa*. BAWDEN and PRIE (1938c) have also found that extracts of healthy tobacco and tomato plants, after precipitation with ammonium sulphate, contain relatively large amounts of proteins which readily sediment when neutral solutions are centrifuged at 16,000 times gravity. These proteins are also found in the sap of infected plants. The failure of WYCKOFF, BISCÆ and STANLEY to demonstrate any sedimentation from healthy plant sap, or from solutions of normal plant protein, in centrifugal fields as great as 180,000 times gravity can probably be explained by their treatment of the plants before expressing the sap. As a routine method the plants are frozen overnight at temperatures around -14°C , because this facilitates clarification. The proteins with high molecular weights found in normal tobacco plants are rendered insoluble if solutions are frozen solid and then thawed. The freezing of the leaves probably destroys most of

these proteins and their precipitation no doubt accounts for the easier clarification after freezing.

In preparing potato virus "X" by means of a high speed centrifuge, LORING and WYCKOFF (1937) obtained substances which sedimented more slowly than the virus fraction. These were not infective and were not always found. No explanation was offered for them, but it seems probable that they were the normal plant proteins with high molecular weights. In their work the plants were frozen before the sap was extracted, and different degrees of freezing may account for the variable results. If the temperature was sufficiently low to freeze the cell sap solid, most of these proteins would be denatured, but if not they would remain in the clarified sap and be detected on centrifugation.

It seems probable that proteins with high molecular weights are normal constituents of a number of different plant species. PRICE and WYCKOFF (1938) have found substances in both healthy cucumber plants, and in those infected with cucumber viruses 3 and 4, which sediment readily on high speed centrifugation and which give all the usual protein reactions. LORING, OSBORN and WYCKOFF (1938) have demonstrated apparently similar substances in peas and beans. These workers were unable to effect any significant purification of pea virus 1 by the high speed centrifugation of infective sap, although they obtained protein preparations behaving in a reasonably homogeneous manner in the high speed centrifuge. The existence of these substances throws some doubt on the purity of virus preparations made solely by high speed centrifugation, and necessitates considerable modification of the earlier tendency to regard anything sedimenting from clarified infective sap as a specific protein characteristic of the infecting virus.

Tobacco ringspot (STANLEY 1939) and alfalfa mosaic (ROSS 1941), two unstable viruses, have been concentrated by ultracentrifugation in the cold. With neither virus, however, was centrifugation alone sufficient to give colourless preparations, even when applied to sap clarified by freezing or by the addition of phosphate. Similarly, preparations of tomato bushy stunt virus (STANLEY 1940) made by centrifugation contain green material that is easily removed by treatment with ammonium sulphate. In combination with other methods of fractionation, differential centrifugation affords an extremely valuable method of purification and is likely to be one that can be applied successfully to most viruses. Used alone, however, it is unlikely to give pure preparations of the unstable viruses which have physical properties similar to those of normal plant proteins and which occur in infective sap at high dilutions. Furthermore, criteria of purity other than apparent homogeneity in the high speed centrifuge are needed before such preparations should be assumed to contain only virus. Proteins readily absorb other materials, so that even in the absence of any other component of high molecular weight, material sedimenting uniformly in intense centrifugal fields is not necessarily pure virus, for it may be an absorption complex.

Yields of purified viruses:— Table 11 gives figures for the yields of

different viruses from infective sap. These can only be taken as indicating the average virus-content of saps, for the actual contents vary widely with different samples. They depend on the age of the inoculated plant, its nutrition and environment as well as the length of time for which it has been infected. For example, the yield of bushy stunt virus from young tomato plants inoculated in the winter may be more than ten times as much as from older plants inoculated in the summer, and SPENCER (1941) has shown that the yields of tobacco mosaic virus are twelve times greater from plants getting liberal supplies of nitrogen than from nitrogen deficient plants. The effect of length of infection is most noticeable with alfalfa mosaic (ROSS 1941a) and tobacco ringspot viruses (STANLEY 1939a), for the virus content of sap from plants infected with these reaches a maximum about 12 days after infection and then fall so radically that it may be reduced to one-fifth or less within a fortnight. In addition to conditions affecting the host plant, the yield of viruses which cause only local lesions, such as tobacco necrosis viruses, also greatly depends on the virus content of the inoculum used. In spite of these variations from preparation to preparation of the same viruses, the figures given in Table 11 clearly show that much greater differences exist between the virus contents of sap from plants infected with different viruses. In general, and as might be expected, the virus content is proportional to the infectivity of the expressed sap and is greatest with viruses such as tobacco mosaic virus with high dilution end points.

In work on purification all workers have used expressed infective sap as their starting material. Some have washed the leaf residues, but as this usually produces only a little extra virus, it has generally been assumed that the residues were substantially virus-free. This is now known to be untrue with several viruses, and the yields recorded in the Table represent only one-half or less of the total virus content of the infected plants. If, after the sap has been expressed, the leaf residue is ground very finely with a roller mill and then extracted with water, considerably more virus is released. From the finely ground residues of plants suffering from tobacco mosaic, bushy stunt or tobacco necrosis, extraction with a volume of water equal to that of the sap gives an extract with approximately the same virus content as the sap itself. How this residual virus was previously bound to the leaf residues, and whether the technique can be generally applied to viruses, is still unknown.

Behaviour of healthy plant sap:— If the detailed methods of purification described for tobacco mosaic or tomato bushy stunt viruses are carried out on sap from healthy plants nothing at all is obtained. Although this is strong presumptive evidence that the virus preparations are free from any constituents of normal plants, it is not proof. For it is possible that such constituents may be carried as contaminants in the virus-precipitates, even though the treatments cause no precipitation when applied to extracts of healthy plants. No substances have been found in healthy plants with properties similar to these viruses. Indeed, it is because of the great differences in stability and properties between these viruses and the normal plant

Table 11:
Approximate weights of different viruses in a litre of infective sap

Virus	Host	Weight of virus
Tobacco mosaic	Turkish tobacco	2.0 gms.
— —	White burley tobacco	2.0
— —	Tomato	1.3
— —	Spinach	0.15
Tomato acuba mosaic	Tomato	1.5
— — —	Turkish tobacco	1.75
Tomato enation mosaic	Tomato	1.5
Masked tobacco mosaic	Turkish tobacco	
Cucumber 3	Cucumber	0.3 —
Cucumber 4	Cucumber	0.3 —
Alfalfa mosaic	Tobacco	0.2 —
Potato "X" S strain	White burley tobacco	0.1 —
— — G strain	White burley tobacco	0.1 —
— — Latent mosaic	<i>Nicotiana glutinosa</i>	0.08 —
	Turkish tobacco	0.04 —
Tomato bushy stunt	Tomato	0.05 —
Tobacco necrosis 1	Tobacco	0.04 —
	Bean	0.01 —
2	Tobacco	0.004 —
	Bean	0.001 —
Tobacco ringspot	Tobacco with symptoms	0.012 —
	Tobacco recovered	0.002 —
Severe etch	<i>Nicotiana glutinosa</i>	0.004 —
— —	Tobacco	0.007 —
Cucumber 1	Tobacco	
Potato "Y"	Tobacco	
Hyoscyamus 3	Tobacco	

proteins that they can be so easily isolated. Other viruses, however, especially the unstable insect-transmitted ones such as potato virus "Y", have properties more nearly resembling those of the normal plant proteins. They precipitate in much the same conditions and are denatured by similar treatments as many of the normal plant proteins. Their isolation is therefore much more difficult and necessitates a detailed knowledge of the behaviour of the constituents of uninfected plants.

Unfortunately, there is little information available concerning the extractable leaf-proteins of the plant species used for the propagation of plant viruses. The early work of CHIBNALL with spinach and other plants showed that the leaf-proteins need gentle handling if denaturation is to be avoided. Stable proteins have been isolated from seeds in crystalline and apparently pure forms, but the leaf-proteins appear to be much more heterogeneous and difficult to purify. The extent

to which proteins of the type described by CHIBNALL (1922, 1924), MENKE (1938) and LUGG (1938) would complicate the isolation of plant viruses cannot be gauged, as sedimentation constants have not been estimated for them. In the early work on the purification of tobacco mosaic virus it was stated that fully infected plants contained several times the total amount of protein found in healthy tobacco plants. These statements were based on the protein content of clarified sap and are now known to be untrue. MARTIN, BALLS and MCKINNEY (1938) were unable to detect any significant differences between the protein content of healthy and mosaic tobacco plants. But there is a difference in the amount of soluble protein. The extent of this difference varies with different samples of sap and with the methods used for clarification. Much of the normal plant protein is extremely unstable, it being rendered insoluble by freezing, heating to 60° C or by moderate acidification. Even addition of sufficient disodium hydrogen phosphate to raise the pH of the sap to 7 causes a precipitate containing proteins to separate. As one or other of these treatments is generally employed to facilitate clarification, and they appear to cause little or no loss of virus, it is obvious that the relative proportions of protein in clarified healthy and infective sap give no indication as to the total protein content of healthy and infected plants.

Expressed sap from fresh tobacco or tomato leaves is difficult to clarify by centrifuging at 3,000 r.p.m. A green precipitate separates but the supernatant is opalescent and darkly coloured. If this is heated to 60° C, acidified below pH 5, or frozen at low temperatures and then thawed, bulky precipitates are produced, which are readily removed by a few minutes centrifuging at 3,000 r.p.m., leaving a clear brown supernatant fluid. If sodium phosphate is added, a smaller precipitate separates leaving a fairly clear supernatant fluid.

The addition of ammonium sulphate to the centrifuged sap causes a bulky precipitate at a quarter saturation. This precipitate differs from those caused by the other treatments for it dissolves readily in water at pH 7 to give a dark-brown viscous solution. These solutions become opalescent when only one-seventh saturated with ammonium sulphate, and after standing for some hours a precipitate separates which can be centrifuged off. A further precipitate can be produced by the addition of a little more salt to the supernatant fluid, and in this way a series of fractions can be obtained differing in their precipitability with ammonium sulphate but similar in all their other properties studied (BAWDEN and PIRIE 1938c). All are soluble in dilute salt solutions at neutrality, but are denatured when the pH is lowered to 5. If kept cold they are reasonably stable, but at room temperature they denature in a few days. They are rendered insoluble by freezing, by heating to over 55° C for a few minutes or by 35% alcohol in the presence of salts. They are also readily destroyed by incubation with commercial trypsin preparations. All the fractions obtained with ammonium sulphate appear to contain proteins with large molecular weights, for when neutral solutions are centrifuged at 14,000 times gravity they deposit isotropic jellies. These can be dissolved in water and again sedimented, when they behave like homo-

geneous preparations. The proteins sediment more slowly than tobacco mosaic virus.

It will be noticed that except for their large molecular weights these normal plant proteins have little in common with the viruses yet purified. They are so unstable that they are removed partly or wholly from infective sap by the preliminary treatments in the purification process. They also differ from the viruses chemically and antigenically. All the viruses that have been obtained in liquid crystalline or crystalline form have been found to be nucleoproteins, and to be active producers of precipitating antibodies when injected intravenously into rabbits. On the other hand, preparations of the normal plant proteins are relatively inactive as producers of precipitating antibodies, and when carefully purified they do not contain significant amounts of carbohydrate or phosphorus. The unstable materials centrifuged from cucumber, *Vigna sinensis* and *N. glutinosa* by PRICE and WYCKOFF (1938, 1939) and from peas and beans by LORING, OSBORN and WYCKOFF (1938), are presumably similar proteins to these found in tobacco and tomato. The latter workers state that their preparation contained a pentose and suggest that it was a nucleoprotein. However, as they give no figures for phosphorus, it is equally possible that the carbohydrate was a contaminant. The material from *V. sinensis* has a sedimentation constant of 51×10^{-13} whereas that from all the other species has a constant of about 75×10^{-13} (PRICE and WYCKOFF 1939).

These unstable proteins with high molecular weights form the greater part of the soluble protein of tobacco and tomato plants. After they have been removed by heating and centrifuging, the greatest differences are found between normal sap and extracts containing the heat-stable viruses, for the supernatant fluids from normal sap now contain little material that can be precipitated with ammonium sulphate whereas those from infective sap contain the viruses. Only a small precipitate is obtained with one-quarter saturated ammonium sulphate from the heated healthy sap. At two-thirds saturation a further precipitate separates. This is apparently a nucleoprotein, for it gives the usual protein colour reactions and contains phosphorus and carbohydrate (BAWDEN and PIRIE 1937a). This protein is unaffected by heating to 80° C, but at 90° C it breaks down to liberate a nucleic acid. It seems to be less resistant than the purified viruses to treatment with acid and salts, for it often denatures after a few precipitations with acid and ammonium sulphate. It is also readily destroyed by proteolytic enzymes which are not known to hydrolyse any viruses other than potato virus "X".

No proteins which show anisotropy of flow or which crystallise in forms similar to tomato bushy stunt or tobacco necrosis viruses have been isolated from healthy tobacco or tomato plants. The phenomenon of anisotropy of flow was first observed in clarified sap from plants suffering from tobacco mosaic by TAKAHASHI and RAWLINS (1933a). Later these workers stated that some samples of healthy tobacco sap also showed the phenomenon, but to a less degree (1933b). BAWDEN and PIRIE (1937a) and LAUFFER and STANLEY (1938) have been unable to confirm this second statement, for they could detect no ani-

sotropy of flow in either clarified healthy sap or in concentrated solutions of normal plants proteins. However, these results do not prove that healthy plants are free from substances giving anisotropy of flow; they merely show that in the samples examined the phenomenon was not detectable. STANLEY (1937*c*) was able to regain tobacco mosaic virus from artificially prepared mixtures containing only one part of virus to 100,000 parts of normal plant material. If normal plants contain any such substances, therefore, it seems that either they must be much less stable than the viruses or occur in extremely minute quantities.

PROPERTIES OF PURIFIED VIRUS PREPARATIONS

Activity:—Most workers have determined the activity of their purified virus preparations by two ways; one by finding the smallest weight of virus that will cause infection and the second by finding the smallest weight of virus that will give a visible precipitate with antiserum. The solid contents of dialysed preparations are most conveniently determined by drying samples of a known volume *in vacuo* while frozen, for the dried viruses are then obtained as light fluffy materials which are easy to handle. The local lesion method is generally used in infectivity tests, 1 cc. of solutions containing various amounts of virus being rubbed as evenly as possible over the leaves of a suitable host. In the serological tests 1 cc. of antiserum at a constant dilution is added to a series of tubes containing 1 cc. of virus solution at different concentrations. The precipitation end point, or serological titre, is taken as the weight of virus in the antigen solution giving the smallest precipitate visible to the naked eye. Neither test is sufficiently reliable to act as anything more than a rough guide for purity, but the results from serological tests are much more constant and reliable than those of infectivity tests, for these vary widely with variations in the host plants or methods of inoculation. Provided the conditions of testing are kept constant, repeat tests on the same virus preparation will give the same precipitation titre, but if the concentration of antiserum is varied the titres will vary. With some viruses, for example those causing tomato bushy stunt and tobacco necrosis, the titres of preparations made at different times are also remarkably constant, but with tobacco mosaic virus there is considerable variation between the titres of different preparations. During the course of purification, changes in the ratio of serological activity to infectivity often occur. Three main reasons seem to cause this. Firstly inhibitors of infectivity may be removed from the sap during purification and this would decrease the ratio. Secondly, virus may be altered so that it ceases to be infective although still remaining serologically active, or thirdly aggregation between particles may occur, either of which would increase the ratio.

In Table 12 are given the serological titres of a few preparations of different viruses together with their infectivities at different dilutions. In general the viruses with anisotropic particles give higher serological titres than those with spherical particles, probably because the more voluminous precipitates given by the former are more easily seen. The serological titres of different strains of tobacco mosaic virus vary; aucuba mosaic virus gives the highest and over a period of years all preparations have given titres of between $1/6$ and $1/10,000,000$. The preparations of tobacco mosaic virus made at Rothamsted in 1936 also gave high titres of between $1/5$ and

Table 12:
Activity of purified virus preparations

Virus	Serological titre	Infectivity at various dilutions. Average number of lesions per leaf.							Host plant
		10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	
Tobacco mosaic	1/8,000,000	416	240	56	6	2	0.5	0.12	<i>Nicotiana glutinosa</i>
- "	1/2,000,000	242	98	29	5	2.1	0.8	---	<i>Phaseolus vulgaris</i>
Aucuba mosaic	1/10,000,000	210	140	34	6	0.4	0	0	<i>N. glutinosa</i>
- "	1/8,000,000	290	82	39	11	3	1	---	<i>N. glutinosa</i>
Enation mosaic	1/6,000,000	352	152	26	2.6	1	0.3	0	<i>N. glutinosa</i>
- "	1/6,000,000	298	72	8	1.2	0.4	0	---	<i>N. glutinosa</i>
Potato "X" Ringspot Strain	1/6,000,000	660	220	42	7	1	0	---	<i>N. Tabacum</i>
- " - "	1/6,000,000	196	38	9	2	2	0.3	---	<i>N. Tabacum</i>
Bushy stunt	1/600,000	105	45	10	3	0.5	0	---	<i>N. glutinosa</i>
- "	1/600,000	86	30	15	1.5	0.2	---	---	<i>N. glutinosa</i>
Tobacco necrosis	1/600,000	---	325	117	60	15	---	---	<i>P. vulgaris</i>
- "	1/600,000	---	119	36	9	---	---	---	<i>P. vulgaris</i>
Tobacco ringspot	1/1,000,000	---	29.8	13.8	0.2	---	---	---	<i>Vigna sinensis</i>
- "	1/1,000,000	---	6.8	2.3	0.1	---	---	---	<i>V. sinensis</i>
Alfalfa mosaic	---	30	2	---	---	---	---	---	<i>P. vulgaris</i>

The serological titres are expressed as the highest dilutions, in grams per cc., at which there was a visible precipitate when 1 cc. of virus solutions was incubated with 1 cc. of diluted antiserum. The dilutions in the infectivity tests are given as grams of protein per cc.

1/8,000,000, but since 1938 it has been rare to get a preparation with a titre exceeding 1/1,000,000. The reason for this change is unknown, but it is possible that mutation and selection in the intervening years has given a culture consisting of different virus strains. The weights of different viruses required to give infection vary from more than one millionth of a gram with alfalfa mosaic virus to less than one ten thousand-millionth of a gram with tobacco mosaic virus. As tobacco mosaic virus weighs about 10^{-17} gram, it is obvious that even the smallest weight giving infection still contains large numbers of particles. Much of the inoculum is no doubt wasted on the leaf surfaces, for the methods of inoculation are relatively crude, but until infection can regularly be produced with less material it cannot be assumed that one virus particle is sufficient to cause infection.

Chemical composition:—When the first edition of this book was prepared the chemical nature of tobacco mosaic virus was still a

Table 13:
Analytical compositions of purified virus preparations

Virus	Carbon	Hydrogen	Nitrogen	Sulphur	Phosphorus	Ash	Carbohydrate
Tobacco mosaic	50%	7.3%	16.5%	0.4%	0.5%	2.0%	2.5%
Aucuba mosaic	51%	7.1%	16.7%	0.4%	0.5%	1.5%	2.5%
Enation mosaic	51%	7.1%	16.7%	0.3%	0.5%	1.0%	2.5%
Cucumber 3 and 4	50%	7.4%	15.6%	0.3%	0.57%	1.5%	2.4%
Potato "X"	49%	7.4%	16.4%		0.45%	2.2%	2.7%
Potato "Y"	50%	—	16.0%		0.4%		3.0%
Alfalfa mosaic	53%	6.7%	16.2%	0.6%	1.4%		9.0%
Bushy stunt	49%	7.7%	16.1%	0.6%	1.4%	3.0%	5.5%
Tobacco necrosis	45%	6.5%	16.3%	1.6%	1.65%	7.0%	6.5%
Tobacco ringspot	51%	7.6%	14.6%	0.4%	4.1%		18.0%

matter of controversy. STANLEY (1935, 1936) originally described it as a globulin and stated that it contained no phosphorus, whereas BAWDEN and PIRIE (1936, 1937a) found both phosphorus and carbohydrate in the form of nucleic acid, and described the virus as a nucleoprotein. BEST (1937c) and LORING (1938) both supported the view that the virus was a nucleoprotein, but this was disputed by STANLEY (1937d) who claimed that the nucleic acid was a contaminant inessential for activity. Fortunately, this controversy has now ceased, for STANLEY (1938c) has agreed with the view that the nucleic acid is an essential part of the virus and all workers are unanimous that this virus is a nucleoprotein. There is thus no need here to reiterate the evidence, given in the first edition of this book, in favour of the view that the nucleic acid is not a contaminant, and it is sufficient to say that no method has yet been found for separating the nucleic acid from the protein without destroying infectivity.

Only a few viruses have yet been sufficiently purified for analytical figures on the preparations to be of any value as a guide to

their chemical constitution. With some of these viruses there has been some variation in the analyses published by different workers at different times. The figures listed in Table 13 are either the most recent or those thought to be most reliable. Although these viruses differ widely from one another in their general properties, it will be seen that their analytical figures differ significantly only in the amounts of phosphorus and carbohydrate. All the viruses yet studied contain these two substances in the form of nucleic acid and, like tobacco mosaic viruses, are nucleoproteins. It would still be premature to assume that all plant viruses are chemically similar, but, as such widely different viruses are so alike, it is probable that many others are also nucleoproteins. The figures for carbon, hydrogen and nitrogen for all the viruses are so similar to those given by proteins in general that they are of no value as a guide to the purity of a preparation. The figures for the phosphorus and carbohydrate, on the other hand, are more characteristic of the different viruses and the ratio between the two is a useful test for purity. The most frequent contaminant of partially purified preparations is rich in carbohydrate, and if the ratio of carbohydrate to phosphorus is greater than 5 to 1 the preparation can usually be further fractionated.

The nucleic acid in all the viruses yet examined is of the same type, but most have not been sufficiently studied to be sure whether they are identical or merely similar. It resembles yeast nucleic acid, and differs from thymus nucleic acid, which is characteristic of nuclei, in that it contains carbohydrate of the ribose type and not a desoxypentose. The ease with which the nucleic acid can be isolated from the different viruses varies, but with each it can be obtained in sufficient quantities to account for so much of the phosphorus and carbohydrate that there is no need to suspect the presence of these in any other form. Preparation of some viruses made by differential ultracentrifugation often give slightly higher carbohydrate contents than those made by other methods, and the ratio of carbohydrate to phosphorus is higher than that for yeast nucleic acid. This suggests that sedimentation alone may be insufficient to remove all adsorbed carbohydrate, although LORING's (1938*b*) claim that other methods of isolation cause slight chemical changes in potato virus "X" which may liberate carbohydrate and reduce infectivity is also possible.

Nucleic acid is readily prepared from tobacco mosaic virus denatured by heat. When neutral solutions containing a trace of salt are heated for a few minutes at 90° C or higher, they become slightly acid and most of the protein coagulates. The addition of more salt causes a further precipitate of protein, and the supernatant fluid now contains little protein but all the phosphorus and carbohydrate. The addition of a mineral acid now causes the nucleic acid to separate as characteristic resinous masses. Alternatively, nucleic acid can be isolated by hydrolysing tobacco mosaic virus with sodium hydroxide at 0° C or by treating with glacial acetic acid or 30% pyridine. When solutions are mixed with five times their volume of glacial acetic acid the nucleic acid separates out while the protein fractions remain in solution. With 30% pyridine the virus solutions go clear and protein fractions can be precipitated by the addition of a little ammonium

sulphate; the addition of acid to the supernatant fluid then precipitates the nucleic acid. About 6% of the weight of the preparation can be isolated as nucleic acid. LORING (1938*a*, 1939*a* and *b*) has studied the nucleic acid in detail and found that it is not identical with yeast nucleic. Dried samples contain about 33% carbon, 4.2% hydrogen, 15% nitrogen, 9% phosphorus and 30% carbohydrate. These figures are similar to those for yeast and triticonucleic acid, and the virus nucleic acid has the same solubility and general properties as yeast nucleic acid. It has a smaller diffusion constant, however, 0.10 sq. cm. per day compared with 0.13 for yeast nucleic acid, suggesting a molecular weight of 37,000 for the virus nucleic acid com-

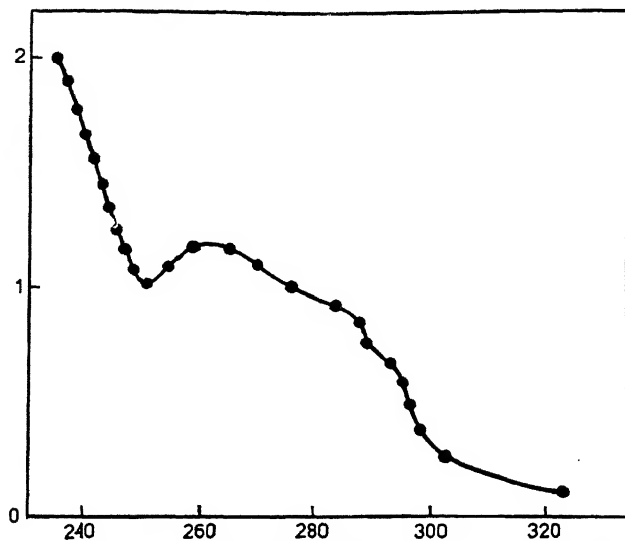


FIG. 28. — Ultra-violet absorption spectra of purified virus preparations. In each of the three curves the abscissa is the wavelength of light in $m\mu$ and the ordinate is the \log_{10} of the amount of light of that wavelength that is incident on a 2 cm. deep layer of solution to the amount of light that is transmitted through the solution. — A: 0.02% solution of tobacco mosaic virus (BAWDEN, F. C. and PIRIE, N. W., 1937, *Proc. Roy. Soc. B.* 123, 274).

pared with 17,000 for yeast nucleic acid. LORING isolated from the hydrolytic products the purines, guanine and adenine, and the pyrimidine, cytosine, as well as the brucine salt of a compound with the nitrogen and phosphorus content of the brucine salt of yeast uridylic acid. The optical activity and solubility of this salt was different from those of the brucine salt of yeast uridylic acid, suggesting that the two were isomeric rather than identical and that the virus nucleic acid contains a new pyrimidine.

Potato virus "X" also breaks down to give protein fractions and nucleic acid when treated with 80% glacial acetic acid or when heated, but the components are not so easily separated as in denatured tobacco mosaic virus. The addition of a little salt to heated preparations causes some protein to precipitate, but most remains in the supernatant fluid from which it can be precipitated in successive fractions

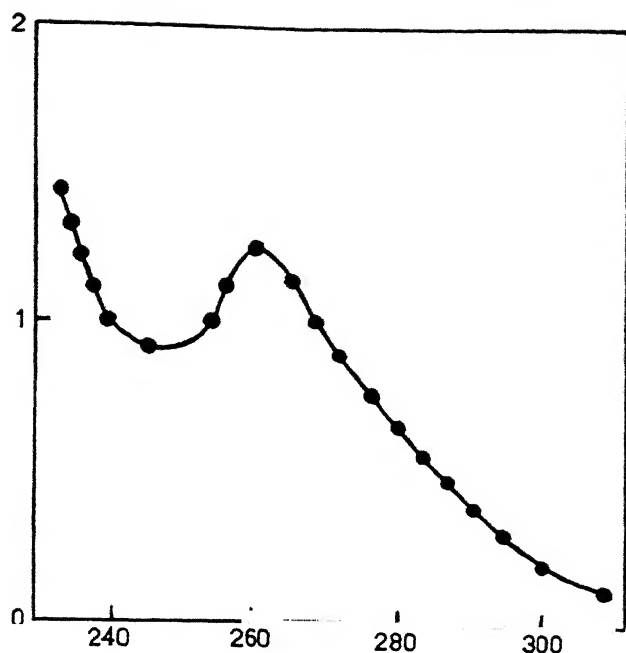


FIG. 28 (*contd.*). — B: 0.012% solution of tomato bushy stunt virus (BAWDEN, F. C. and PIRIE, N. W., 1938, *Brit. J. exp. Path.*, 19, 251).

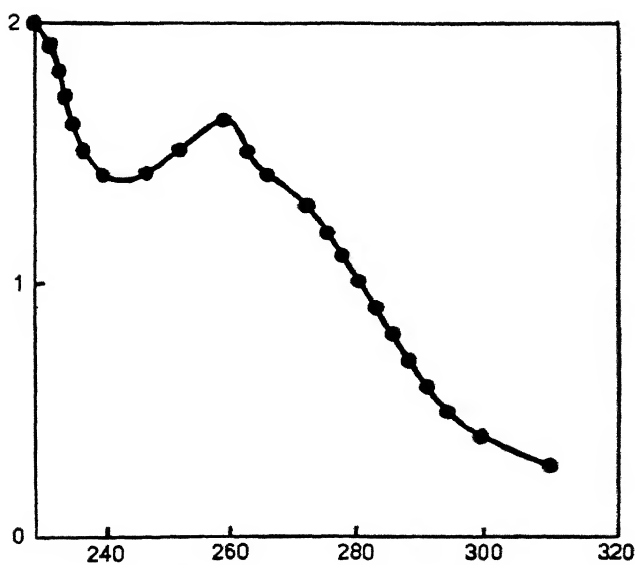


FIG. 28 (*contd.*). — C: 0.013% solution of tobacco necrosis virus (PIRIE, N. W., SMITH, K. M., SPOONER, E. T. C. and MACCLEM-ENT, D., 1938, *Parasitology* 30, 543).

by the gradual addition of acid. All the protein precipitates when the pH has been brought to 4, but the fractions separating at the lower values contain phosphorus. The addition of a strong mineral acid to the fluid now produces a precipitate of nucleic acid, which can be further purified by dissolving in dilute soda and again precipitating with acid. With glacial acetic acid the best results are got by adding four volumes to the tightly packed precipitates produced by the prolonged centrifugation of virus solutions at pH 4.5, when the protein fractions dissolve leaving a residue of crude nucleic acid. This appears similar to the nucleic acid isolated from tobacco mosaic virus, but it has not been examined in any detail. It contains ribose and guanine and has the same solubility as yeast nucleic acid. Tomato bushy stunt virus (BAWDEN and PIRIE 1938b; STANLEY 1940), alfalfa mosaic virus (ROSS 1941) and tobacco ringspot virus (STANLEY 1939) have also been found to contain nucleic acids of the yeast type. The other viruses also give negative Dische and Feulgen reactions, so that it is probable that they also contain nucleic acid of the yeast type.

The fact that preparations of different viruses contain widely different amounts of phosphorus and carbohydrate suggests that they have different contents of nucleic acid. This suggestion is supported by comparing the ultra-violet absorption spectra of solutions of the viruses. As solutions of all carefully purified viruses are colourless, there is little absorption of visible light. In the ultra-violet range, the absorption increases to a maximum at 260 m μ , followed by a minimum at 250 m μ and general absorption at shorter wavelengths; 260 m μ is the region generally associated with absorption by nucleic acid and other substances containing purines. The absorption of solutions of different viruses at 260 m μ is correlated with their contents of phosphorus and carbohydrate (Fig. 28); viruses, such as tobacco mosaic and potato "X", with 0.5% phosphorus absorb less strongly at this wavelength than bushy stunt or tobacco necrosis, which have more than twice as much phosphorus, and these again absorb less strongly than tobacco ringspot virus which has 4% of phosphorus. This is reflected in the amounts of nucleic acid that can be isolated from denatured preparations. About 6% of the original virus can be obtained from tobacco mosaic virus as a protein-free nucleic acid, about 15% from bushy stunt virus and 40% from tobacco ringspot virus. The figures published at different times for the phosphorus content of tobacco ringspot virus have varied widely. STANLEY and LORING (1938) gave a first figure of 1.8% and a second of 3.2% (1939), while STANLEY (1939) gave 4.1% as quoted in Table 13. These different figures have all been given for preparations prepared solely by differential centrifugation, so that if the figure of 4.1% is taken as the real phosphorus content even this method of isolation on its own seems often to lead to separation of the nucleic acid from the protein. Precipitation with ammonium sulphate inactivates tobacco ringspot virus and causes a reduction in the phosphorus content, presumably by splitting off some nucleic acid. Thus this virus seems to resemble sperm nucleoproteins in the ease with which nucleic acid is dissociated from protein. By contrast the nucleic acid in tomato

bushy stunt virus is so firmly attached to the protein fraction that methods of denaturation which readily separate the two components of tobacco mosaic virus fail to cause dissociation. For example, the coagulum that separates from boiled solutions containing salt carries all the nucleic acid with it, and treating the virus with cold soda extracts only part of the nucleic acid. The nucleic acid can be extracted from the heat-denatured virus by boiling with ammonia and precipitating with acid, or by digesting the coagulum with trypsin preparations that contain no nuclease. If bushy stunt virus is treated with warm acetic acid or with surface active agents such as sodium dodecyl sulphate, the addition of salts causes the separation of protein free from phosphorus, and the nucleic acid can then be precipitated from the supernatant fluid by the addition of acid.

As yet little work has been done on the composition of the protein fraction of the viruses, and this only on tobacco mosaic virus. When the virus is denatured by heat a series of protein fractions can be obtained, some of which are soluble in dilute salt solutions at neutrality and others insoluble. The soluble fractions can be precipitated by reducing the pH to 4. No significant amounts of material can be extracted from these proteins by prolonged treatment with ether, acetone, alcohol or pyridine. The fractions are readily hydrolysed by proteolytic enzymes which have little or no effect on the intact virus particle; similarly the free nucleic acid is hydrolysed by nuclease which has no effect on the virus. When hydrolysed by boiling with acid they break down like other proteins to give amino-acids. ROSS (1941*b*) states that tobacco mosaic virus contains 3.9% tyrosine, 4.5% tryptophane, 4.7% proline, 9.0% arginine, 6.7% phenylalanine, 6.4% serine, 5.3% threonine, and 0.68% cysteine or cystine. No glycine or histidine were found. ROSS (1940) considers that the content of cysteine is sufficient to account for all the sulphur, although earlier ROSS and STANLEY (1939) stated that the virus contains both methionine and sulphate sulphur. The active virus does not give a positive nitroprusside test, but after denaturation there are free sulphhydryl groups.

Except for tobacco ringspot virus and cucumber virus 3 and 4, all the other viruses purified have also given positive nitroprusside tests after denaturation. KNIGHT and STANLEY (1941) also state that cucumber viruses 3 and 4 contain four times as much sulphur as tobacco mosaic virus, so that the sulphur content of these serologically related viruses differs both qualitatively and quantitatively. These cucumber viruses also differ significantly from tobacco mosaic in their contents of certain aromatic amino-acids (KNIGHT and STANLEY 1941*a*). Tobacco mosaic virus and four other strains closely related serologically all contain 3.8% tyrosine, 4.5% tryptophane and 6.0% phenylalanine, whereas cucumber viruses 3 and 4 contain 3.8% tyrosine, 1.4% tryptophane and 10.2% phenylalanine. A virus obtained from plantains by HOLMES (1941), which is also remotely related serologically to tobacco mosaic virus, shows differences of a similar type, for this contains 6.4% tyrosine, 3.5% tryptophane and 4.3% phenylalanine.

Using sodium dodecyl sulphate to disrupt tobacco mosaic virus,

SREENIVASAYA and PRIE (1938) have obtained protein fractions that remain soluble and can be fractionated. After incubation with 1% sodium dodecyl sulphate for some hours at pH 8, virus solutions lose all their characteristic properties, and nothing sediments from them in a centrifugal field of 16,000 times gravity, showing that the large particles have been broken down. After removing the sodium dodecyl sulphate by dialysis, all the protein can be precipitated by the addition of about one-tenth of a volume of saturated ammonium sulphate. Provided that the pH is above 8, the nucleic acid remains in the supernatant fluid, from which it can be precipitated by the addition of acid. When freed from nucleic acid, the protein precipitated with ammonium sulphate forms a clear, gelatinous sediment when centrifuged. This can be redissolved in water and again precipitated with ammonium sulphate, but it is much less resistant to these treatments than the intact virus. If the precipitation is repeated many times the protein changes partly into an insoluble fraction and partly into one that is precipitable with only 2% saturated ammonium sulphate solution. A similar denaturation takes place if the protein is kept for a few days at room temperature or if it is acidified. The insoluble portion resembles the coagulum produced by heating the virus in that it is susceptible to tryptic digestion, whereas the soluble portion resembles the intact virus in resisting attack by trypsin. A number of attempts to fractionate these soluble proteins has failed to show any appreciable heterogeneity. They are much less stable than the intact virus. As they neither sediment in a high speed centrifuge nor pass through cellophane membranes, their molecules are much smaller than the virus particles and have weights more comparable with those commonly found for proteins. Thus the large stable particle of tobacco mosaic virus can be broken down into a non-protein part (the nucleic acid) and a protein part, which is less stable than the virus and contains many similar or identical molecules of smaller molecular weights. How the protein and nucleic acid are combined in the virus particle is unknown, but the production of relatively homogeneous protein preparations from the denatured virus, and the regularity of the X-ray pattern (BERNAL and FANKUCHEN 1941), suggest that the particle is built up from many small protein units bound together by molecules of nucleic acid.

Centrifugal studies on preparations of tobacco mosaic virus inactivated by exposure to acid and alkali also show that the virus can be disrupted in steps to give soluble products with widely different molecular sizes. ERIKSSON-QUENSEL and SVEDBERG (1936) found that the virus particles split after a short time at pH 9.8, the components of the preparation then sedimenting only a little more slowly than the intact virus. Above pH 11 there was a much more striking effect, for the soluble components then sedimented at about the same speed as proteins such as egg albumin with small molecular weights. WYCKOFF (1937) has also found that the more severe the treatment with acid or alkali the more slowly the breakdown products sediment. During inactivation with alkali, he obtained products varying in size from those which sedimented only slightly more slowly than the intact virus to those which did not sediment at all in a centrifugal

field of 16,000 times gravity. When disrupted by alkali the opalescent virus preparations become clear and cease to show anisotropy of flow, indicating that the smaller breakdown products are more nearly spherical in shape than the intact virus particle. Because of this difference in shape it is impossible to compare the sizes of these breakdown products directly with that of the intact virus particle on the measurements of their rate of sedimentation, but the results indicate strongly that the virus can be broken down in a number of steps to liberate fragments greatly differing in molecular size. Some of these appear to be sufficiently stable to be isolated and it is probable that more detailed studies of them will supply useful information on the internal structure of the virus particles. Preliminary studies with tomato bushy stunt virus (McFARLANE and KEKWICK 1938) have given similar results to tobacco mosaic virus. After inactivation with acid and alkali, components appear in the preparation which sediment more slowly than the intact virus, but fewer components have been found than in tobacco mosaic virus and the smallest still sediments readily in a centrifugal field of 20,000 times gravity.

Although preparations of tobacco mosaic virus have now been broken down by many different treatments, no constituents other than protein, nucleic acid and ash have been detected. From the evidence at present available it seems probable that the large virus particles are built up from the union with nucleic acid of a number of similar or identical proteins with more usual molecular weights. The other purified virus preparations have not yet been examined in such detail, but they also seem to contain little other than nucleic acid and protein. LORING (1938*b*) states that his preparations of potato virus "X" have too high a carbohydrate content for them to be only nucleoprotein, but there is not yet sufficient evidence to regard the extra carbohydrate as an essential constituent of the virus. If there are any other constituents of these virus preparations they must be minor ones, and it is in their apparent chemical simplicity that the viruses differ most definitely from any recognised organisms. Bacteria, of course, have a high protein content, but in addition they contain fats, carbohydrates and a number of other diffusible substances. In sharp contrast, these viruses seem to consist solely of nucleoprotein; they do not merely contain nucleoprotein, but they appear to be nucleoprotein. And from their chemical composition they resemble constituents of organisms rather than any recognised organisms themselves.

All protein preparations leave an ash when they are burnt, but the ash contents of the purified virus preparations, especially those of tomato bushy stunt and tobacco necrosis viruses, are considerably greater than is usual with other proteins. The percentages of ash given in Table 15 are the weights of the residues remaining after preparations at pH 7 have been burnt in a current of air or oxygen for the determination of carbon and hydrogen contents. As all the viruses used have isoelectric points at pH 4.5 or below, some of the ash will be derived from the NaOH used for neutralisation. But even after prolonged dialysis, or electrodialysis, at the isoelectric points, the virus preparations still give unusually high percentages of ash. However, it is probable that only a small part of this ash will be the

actual metal content of the virus, for much of it will represent the phosphorus of the nucleic acid which will be converted to metaphosphate during the burning (PIRIE and others 1938). This also explains the greater ash obtained from tomato bushy stunt and tobacco necrosis viruses than from tobacco mosaic virus and potato virus "X". The identity of the metals in the preparations of any of the viruses has not yet been determined.

Specific gravity:— The first measurements on the specific gravity of tobacco mosaic virus were made by ERIKSSON-QUENSEL and SVEDBERG (1936). They determined the specific volume of the material from one of STANLEY's early preparations and found it to be 0.646, giving a specific gravity of 1.55. This differs considerably from the figure of 1.33 usually found for proteins and has not been confirmed by later workers. BAWDEN and PIRIE (1937*a*) used three different methods for measuring the specific gravity and obtained values varying between 1.3 and 1.37. The lower values were given by flotation methods in which either the dried virus was equilibrated in mixtures of nitrobenzene and dichlorobenzene or the material precipitated from purified solutions with ammonium sulphate was equilibrated in solutions of sucrose and ammonium sulphate. The higher values were given by pycnometric methods in which the specific volume of the virus was determined from the measurements of the specific gravity of solutions of known concentration. The figures agree well with those usually found for proteins and the differences between the values as determined by the different methods also agree well with similar observations on other proteins. No differences were found between the values for the specific gravity of three strains of tobacco mosaic virus. The values 0.77 and 1.3 for the specific volume and specific gravity have been confirmed by STANLEY (1938*a*) using a pycnometer equilibrated in toluene and butyl alcohol. Similar values have also been found for tomato bushy stunt virus (McFARLANE and KEKWICK 1938), potato virus "X" (LORING 1938*b*) and tobacco necrosis virus (PIRIE and others 1938). ROSS (1941) gives the specific gravity of alfalfa mosaic virus as 1.48, but the preparations on which the measurements were made were not homogeneous. Tobacco ringspot virus has a much higher specific gravity than the other viruses, as would be expected if it is 40% nucleic acid. STANLEY (1939) gives the specific volume as 0.636, corresponding to a specific gravity of 1.57.

The specific gravity is important as it is included in the formula used for translating sedimentation constants into molecular weights. The methods described above, however, give the specific gravity of material in the dried state and may not give the true specific gravity of the virus particles in solution. For it is possible that the virus particles in solution take up water, so reducing their effective density below that of the dried products. From X-ray measurements on tomato bushy stunt virus, BERNAL, FANKUCHEN and RILEY (1938) suggest that the particles may be associated with as much as 63% of water, so that their density in solution will be only 1.1 instead of the value of 1.3 found for the dried material.

Precipitation and isoelectric points:— All the viruses that have yet

been studied are easily salted out of neutral solutions, probably because of their large particle sizes. They are all precipitated, when in the partly purified state, by from one-eighth to one-quarter saturated ammonium sulphate solution. The exact amount required, however, varies with the different viruses and also with different preparations of the same virus, depending upon the pH value, the purity, and the temperature. Neutral solutions of all the viruses are opalescent, the amount of opalescence varying with the concentration and, in preparations of tobacco mosaic virus and potato virus "X", with the purification methods used. On the addition of salts the fluids become opaque, but solutions of the different viruses behave differently.

After addition of a little ammonium sulphate, solutions of tobacco mosaic virus become gelatinous and develop an intense satin-like sheen. When they are only one-eighth saturated with ammonium sulphate the fluids may give a precipitate which can be centrifuged off at 3,500 r.p.m., but the material sediments more easily if the concentration of ammonium sulphate is raised to one-fifth or one-sixth of saturation. Solutions of cucumber viruses 3 and 4 between pH 6 and 7 are more opalescent than those of tobacco mosaic virus, and precipitates are formed with slightly less salt. The ammonium sulphate precipitates of all the tobacco mosaic type viruses are similar in appearance. They show a pronounced sheen, especially if stirred, and are composed of microscopically visible needles averaging about $40\ \mu$ long and $0.4\ \mu$ wide.

Neutral solutions of potato virus "X" do not precipitate until the concentration of ammonium sulphate is raised to over one-sixth of saturation, but once precipitated in this way the virus will not redissolve until the concentration is reduced to about one-twentieth of saturation. Although neutral solutions of potato virus "X" resemble those of tobacco mosaic in appearance and both show the phenomenon of anisotropy of flow to about the same degree, the precipitates of the two differ greatly. Those of potato virus "X" do not show a sheen and they are extremely difficult to see, although they are readily thrown down by centrifuging at 3,500 r.p.m. When examined microscopically they are found to be amorphous, and if the precipitated material is shaken between crossed polaroid plates it shows no anisotropy of flow. By contrast, the intensity of anisotropy of flow shown by tobacco mosaic virus is increased by precipitation with ammonium sulphate, suspensions of the precipitated needles showing the phenomenon at greater dilutions than the virus in solution. With insufficient salt to produce precipitation, potato virus "X" preparations more nearly resemble those of tobacco mosaic virus, for in about one-tenth saturated ammonium sulphate solution the fluids develop a slight sheen and show increased anisotropy of flow.

With tomato bushy stunt and tobacco necrosis virus preparations the type of precipitate obtained with ammonium sulphate depends upon the conditions of precipitation. The solutions show a definite increase in opalescence when they are from one-sixth to one-fifth saturated with the salt at 20°C . If more is added, or if the fluids

are allowed to stand for some time at this temperature, a white precipitate separates composed entirely of amorphous material. On the other hand, if the opalescent fluids are cooled to 0°C the amorphous material dissolves, and after some hours crystalline precipitates begin to separate. Fully crystalline preparations are only obtained when the precipitation with ammonium sulphate is carried out slowly with dilute solutions at 0°C . These two viruses both have different solubilities in the two states. In the amorphous form they readily dissolve in water and their solubility in dilute ammonium sulphate solutions is greater at 0°C than at room temperature. In the crystalline state they dissolve more slowly in water, and they are no more soluble in dilute ammonium sulphate solution at 0°C than they are at room temperature.

When acid is added to purified preparations, the behaviour of the individual viruses differs even more than with ammonium sulphate. With tobacco mosaic viruses the result obtained depends on the salt content of the preparations. On the addition of acid to solutions of tobacco mosaic virus with a salt content of $\text{M}/50$ they develop a sheen and become increasingly opaque, until at pH 3.3 the whole of the virus is precipitated and can be removed by centrifuging at 3,500 r.p.m. In the absence of salts, precipitation occurs with less acid and is optimal at pH 4.2, but the fluids do not show such a pronounced sheen and to remove the precipitate the fluids need a longer period of centrifugation at 3,500 r.p.m. If more acid is added the virus again dissolves. In the absence of salts, the fluids become clear at about pH 3.5, and in the presence of salts at about pH 3. Enation mosaic virus behaves in essentially the same manner as tobacco mosaic virus, giving the same optimal precipitation points with acid in the presence and absence of salts. *Aucuba* mosaic virus, however, behaves slightly differently. In the presence of salts it precipitates optimally at pH 3.6 and in their absence at about pH 4.5. Cucumber viruses 3 and 4, which are serologically related to tobacco mosaic virus but have hosts with more alkaline sap, differ even more widely in their precipitability with acid. In the presence of salts, they precipitate optimally at about pH 4.8. In the absence of salts, they are not easily sedimented by centrifuging at 3,500 r.p.m. at any pH value, but they are often less soluble at pH 5.5 than at pH 4.8.

Measurements of the isoelectric points of tobacco mosaic and *aucuba* mosaic viruses by electrophoresis experiments have also given results that vary with the salt content of the preparations. ERIKSSON-QUENSEL and SVEDBERG (1936) found that purified preparations migrated in a uniform manner in an electric field, and in acetate buffer the isoelectric point was pH 3.49. In similar experiments with suspensions of the virus in solutions of NaCl and $(\text{NH}_4)_2\text{SO}_4$, LORING and STANLEY (1937) found isoelectric points between pH 3.2 and 3.35; they state that the actual value varied slightly with the salt content of the buffer mixture used and when acetic acid was present it was raised by from 0.1 to 0.3 of a pH unit. No electrophoresis measurements have been made on salt-free preparations, but it seems that the pH value at which the virus is insoluble is the same as that at which

it does not migrate in an electric field. And it appears that this value, as with some other proteins (*cf.* ADAIR 1937¹), can be varied by altering the concentration of electrolytes.

It is probable that the virus is actually isoionic, that is to say contains equivalent amounts of anions and cations, at the higher pH value at which it is insoluble in the absence of salts. Evidence for this has been obtained by determining the anions and cations in union with the virus. First the preparations were freed from all diffusible ions other than NH_4^+ and Cl^- by repeated precipitations from $N/20$ NH_4Cl with HCl and redissolving in NH_4OH . The precipitated virus was then washed free from salt by repeated shaking up in water, centrifuging and decanting of the supernatant fluids. As the salt content was reduced the virus tended to dissolve at pH 3.3, but this was checked by adding NH_4OH . The washing with water and the addition of NH_4OH to bring the virus to its optimal precipitation point at the corresponding salt content, was repeated until the virus no longer tended to dissolve when washed with water. The pH had then been raised to 4.2. The washing was then continued until the supernatant fluid contained insignificant amounts of ammonium and chloride ions. The NH_4^+ and Cl^- in union with the virus were then liberated by dissolving the virus in $N/5$ $NaNO_3$. Sufficient HNO_3 was then added to bring the pH to 3.3, when the virus was removed by centrifugation, and the NH_4^+ and Cl^- in the supernatant fluid was determined by titration against HNO_3 and $NaCNS$ respectively. As these ions were found in equivalent amounts, it follows that the virus was substantially isoionic at pH 4.2. It seems, therefore, that in the presence of electrolytes the virus can combine with an excess of anions, but that these are in some way prevented from taking part in electrokinetic movement and from allowing the virus to dissolve.

Precipitates of potato virus "X" produced by acid are amorphous and the suspensions show no anisotropy of flow. The virus precipitates optimally from purified preparations at about pH 4.5, but unlike tobacco mosaic virus a preparation does not precipitate completely at any one pH value. After long periods of centrifugation at pH 4.5 and 3,500 r.p.m. some remains in solution. This usually precipitates at either pH 4 or pH 5. In electrophoresis experiments with collodion particles coated with potato virus "X", LORING (1938^b) found that the isoelectric point obtained varied considerably with the previous treatment of the preparation. Freshly-prepared samples made by high speed centrifugation were isoelectric at about pH 3.7 in buffer solutions containing phthalate, phosphate and borate. Samples which had been precipitated with ammonium sulphate or potassium citrate were isoelectric in the same conditions at about pH 4.4 and others treated with 3% dibasic phosphate at pH 7 gave isoelectric points of about pH 5. He suggests that contact with strong electrolytes is responsible for the great variations in cataphoretic behaviour. When solutions of potato virus "Y" are treated with acid or salt, precipitates are formed in much the same conditions and with similar appearances as those of potato virus "X" (BAWDEN and PIRIE 1939). Alfalfa mosaic virus is also precipitated

by acid and its isoelectric point, as indicated by measurements of minimum solubility, is pH 4.6 (Ross 1941).

Tomato bushy stunt and tobacco necrosis viruses behave differently with acid, for these are soluble over the whole pH range in which they are stable. The addition of acid, therefore, produces no visible effect and apparently causes no aggregation of the particles at all, for bushy stunt virus sediments at approximately the

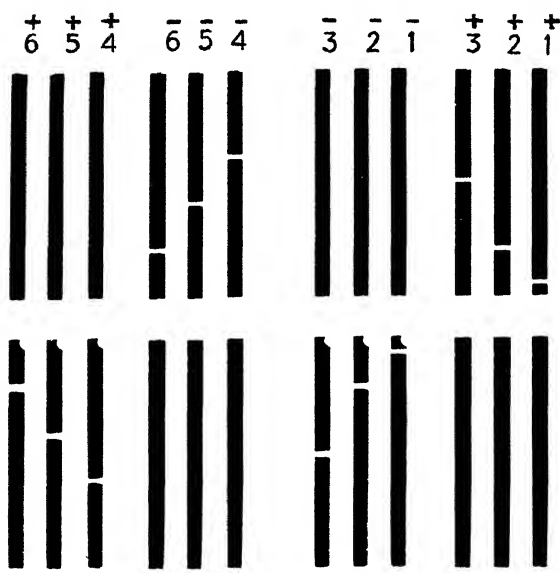


FIG. 29. — Electrophoretic migration of tomato bushy stunt virus in 0.02 M acetate buffer at pH 6.2. Potential gradient 9.5 V./cm. A single sharp boundary is present in all the exposures. (McFARLANE, A. S. and KEKWICK, R. A., 1938, *Biochem. J.* 32, 1607).

same rate in a high speed centrifuge at all pH values between 2.4 and 8.7. McFARLANE and KEKWICK (1938) have made electrophoretic mobility measurements on solutions of tomato bushy stunt virus, and found the isoelectric point to be pH 4.1. Preparations of this virus migrate in an electric field in a manner characteristic of a homogeneous substance, a single sharp boundary being maintained at all pH values (Fig. 29). The isoelectric point of tobacco ringspot virus, determined by cataphoresis, is pH 4.7; at this pH value the virus is unstable, but STANLEY (1939) gives no information about its solubility.

OPTICAL PROPERTIES OF PURIFIED VIRUS PREPARATIONS

General considerations:—In describing the optical properties of purified virus preparations it is necessary to use a number of phrases that are probably unfamiliar to many biologists. A brief description of these and of the behaviour of polarised light is therefore given. Greater details and the theory underlying these processes can be found in any textbook on optics.

Ordinary light is conveniently regarded as a series of electrical disturbances vibrating in all planes at right angles to the direction of transmission. In passing through a Nicol prism the light is resolved into two beams at right angles to one another and in each of which the vibrations are all in one plane. In the Nicol prism two pieces of Iceland spar are cemented together in such a manner that one beam is transmitted through the prism whereas the other is reflected off the cement layer and is absorbed on the blackened side of the prism. Polarising crystals such as tourmaline, or suspensions of polarising crystals such as "Polaroid", similarly resolve light into two beams, one of which is transmitted whereas the other is absorbed in the body of the crystals. Thus the electrical disturbances in the beam of light transmitted by a Nicol prism or a polarising crystal are all in one plane. Such a beam of light is said to be plane polarised. If a beam of plane polarised light is passed through a second polariser with its plane of polarisation parallel to that of the first, the light is transmitted. On the other hand, if the second polariser is rotated through an angle of 90° , *i.e.*, if the two are "crossed", no light is transmitted, for the vibrations in the one plane transmitted by the first are now eliminated by the second.

Some substances transmit light in such a manner that it is conveniently regarded as consisting of two plane polarised rays. These are at right angles to each other and are known as the fast and slow rays because they travel through the substances with different velocities. Substances transmitting light in this manner are said to be doubly refracting, birefringent, or optically anisotropic, and the light they transmit is said to be elliptically polarised. All crystals are doubly refracting except those belonging to the regular, isometric, or cubic, system. Plane polarised light, like ordinary light, is changed into elliptically polarised light by passage through a doubly refracting crystal, unless the crystal is in such a position that one of its transmission planes is parallel to the plane of polarisation of the incident light; the plane polarised light is then transmitted unchanged. As elliptically polarised light has two sets of disturbances at right angles to each other, it cannot be extinguished completely by a Nicol prism (if the position of the prism is such that the fast ray is eliminated, then

the slow ray will be transmitted, and *vice versa*). It is therefore easily distinguished from plane polarised light.

If a single crystal between crossed Nicol prisms is rotated through 360° , in four positions at right angles to one another, where one of the transmission planes of the crystal is parallel with the plane of polarisation of the first prism, no light will be transmitted through the second prism. Mid-way between these so-called extinction positions the amount of light transmitted through the second prism will be at a maximum. Thus the crystal will be visible at all positions except the four extinction positions. If a preparation containing many separate crystals arranged at random is rotated through 360° , each crystal will show four extinction positions, but light will be transmitted through the second prism at all positions because the transmission planes of individual crystals will be parallel with the plane of polarisation at different positions.

Amorphous materials, *i.e.*, materials in which the constituents are arranged quite at random, are not birefringent. Nor are crystals belonging to the cubic system, but all other crystals and other systems possessing some degree of orderly molecular arrangement are birefringent. Many fibrous substances, typified by cellulose, hair and muscle, are highly birefringent, although they cannot be obtained in the form of crystals. Most liquids are quite amorphous and therefore optically isotropic, but in some the arrangement is sufficiently orderly for them to be anisotropic. If they are birefringent when in a state of rest they are called liquid crystals, and it is generally accepted that liquid crystals are formed only by asymmetrical particles which arrange themselves parallel to one another. If the liquids are isotropic when stationary, but become birefringent when made to flow or when a shearing force is applied, they are said to show the phenomenon of anisotropy of flow; this is also known as stream double refraction or flow birefringence (Fig. 30).

Anisotropy of flow can result from one or more of a number of causes. If substances which are normally optically isotropic, such as glass and some other highly viscous gel-like fluids, become deformed when made to flow, they are subjected to internal stresses and become birefringent at points of strain. This phenomenon is known as the photoelastic effect. In less viscous fluids anisotropy of flow results from the presence of small asymmetrical particles. When the fluid is at rest Brownian movement keeps the particles distributed at random, but when the fluid is made to flow they become orientated in a direction parallel to the lines of flow, in the same manner as logs floating down a stream. The particles may be rod- or plate-like and may themselves be optically isotropic or anisotropic, but the more asymmetrical they are the more easily will they be orientated and show anisotropy of flow. Single anisotropic particles small relative to the wavelength of light will not produce a visible effect between crossed Nicol prisms, because they are too small to produce a detectable difference between the fast and slow rays. When arranged at random their individual effects will be compensatory instead of additive, so that a statistical optical isotropy results. If the asymmetrical particles are themselves optically isotropic, the double refraction obtained when

they are orientated by flow results entirely from the difference between the refractive indices of the particles and the surrounding medium. On the other hand, if they are optically anisotropic, when they are orientated by flowing there will be an additional effect resulting from their intrinsic birefringence.

Anisotropy of flow and the layering phenomenon:— Anisotropy of flow in connection with viruses was first described by TAKAHASHI



FIG. 30. — Anisotropy of flow. Wake of a goldfish swimming in a dilute solution of tobacco mosaic virus, photographed between crossed Nicol prisms. Where the fluid is stationary no light is transmitted, but where the rod-shaped particles are orientated by flow movements the fluid becomes birefringent. (BAWDEN, F. C., PIRIE, N. W., BERNAL, J. D., and FANKUCHEN, I., 1936, *Nature* 138, 1051).

and RAWLINS (1933*a*), who noticed it in clarified sap from plants infected with tobacco mosaic virus. They suggested that the virus particles were responsible for the phenomenon, but later (1933*b*) cast some doubt on this by stating that sap from some healthy tobacco plants also showed it, although to a lesser degree. TAKAHASHI and RAWLINS concluded that infective sap showed this phenomenon because it contained rod-shaped particles. They eliminated the possibility of the particles being plate-like by showing that the entire

path of the flowing infective sap was anisotropic, whereas only the edges of the stream were anisotropic when suspensions of particles known to be plates (ferric oxide sol) were made to flow. This conclusion has been fully substantiated by further work.

Solutions of purified tobacco mosaic virus show anisotropy of flow at such high dilutions that their viscosity does not differ significantly from that of water. It is unlikely, therefore, that it results from the photoelastic effect. Also, if it were produced by internal stresses, it should disappear rapidly when the shearing force is eliminated. LAUFFER and STANLEY (1938), however, have shown that when a solution of tobacco mosaic virus is allowed to flow from a pipette into the air, the birefringence persists almost unchanged for some time after the fluid leaves the pipette, *i.e.*, after the shearing force has been greatly reduced.

This agrees well with the view that the phenomenon results from the orientation of rods, for after orientation by flow through the pipette some time would be needed before these would revert to the random state. Other properties of purified preparations of tobacco mosaic virus also indicate the presence of greatly elongated particles. These are their intense sheen (*cf.* the sheen shown by solutions of long-chain soap molecules), their ability to form dilute liquid crystalline solutions, and the fact that X-ray measurements have failed to reveal the length of the particles although distances of nearly ten times the width have been measured (BERNAL and FANKUCHEN 1939).

Evidence that the rod-shaped particles responsible for the anisotropy of flow shown by infective tobacco mosaic sap were the virus particles themselves was first supplied by BAWDEN, PIRIE, BERNAL and FANKUCHEN (1936). It was found that highly purified preparations of the virus, if sufficiently concentrated, separated into two liquid layers

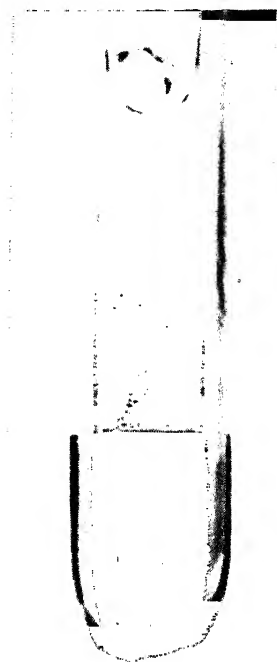


FIG. 31. — Photograph, in polarised light, of a 2% solution of purified tobacco mosaic virus which has been allowed to settle into layers. The lower layer is spontaneously birefringent. The upper layer is birefringent only when a shearing force is applied. (BAWDEN, F. C. and PIRIE, N. W., 1937, *Proc. R. Soc. B.* 223, 274).

when allowed to stand undisturbed. The two layers were found to have different solid contents and different appearances, both when viewed by ordinary light and between crossed Nicol prisms (Fig. 31). The upper layer is the more dilute and by transmitted light is definitely opalescent, while the bottom layer, in spite of its greater solid content, is usually water clear. This at once indicates the greater optical inhomogeneity of the upper layer resulting from the lack of any regular arrangement of the particles. The lower layer in newly separated solutions has an intense sheen when viewed by reflected light. This effect probably results from incomplete separation of the two phases, it

being caused by the presence of small droplets of top layer fluid suspended in the bottom layer. If the solutions are left undisturbed for some months, or if they are centrifuged at 16,000 r.p.m. for 30 minutes, complete separation is obtained and the resulting lower layer is perfectly clear whether viewed by transmitted or reflected light. When the layered solutions are examined between crossed Nicol prisms, the lower layer is seen to be spontaneously birefringent. The stationary upper layer is not liquid crystalline, but if gently agitated it shows the phenomenon of anisotropy of flow strongly. These phenomena are illustrated in Figs. 30 and 31. The first stage in the formation of layers in an originally homogeneous virus solution is the production of small birefringent droplets or tactoids (Fig. 32). These spindle-shaped bodies are formed as a result of the competing tendencies of surface tension to form a spherical aggregate and mutual orientation of particles tending to produce a linear one. Other fluids containing rods, for example, solutions of vanadium pentoxide, form similar tactoids, but with these the separation of a liquid crystalline layer has not been described. In each tactoid the long particles are arranged parallel one to another, and because the concentration of virus in it is slightly greater than in the surrounding isotropic fluid, it has a higher specific gravity and so falls to the bottom of the tube.

The formation and settling of tactoids continues until the concentration of virus in the upper layer is reduced to a level at which the rod-shaped particles have sufficient space to rotate freely in all directions. Brownian movement will then keep them distributed at random, so that the final upper-layer solution is isotropic unless some shearing force is applied to orientate the rods. In the more concentrated droplets which go to form the bottom layer, however, the particles are so close together that rotation about the two shorter axes becomes impossible, and, in order to be accommodated in the available space, the particles are forced to lie parallel one to another. The bottom layer consists of a collection of regions arranged at random in three dimensions, but inside each region there is this parallel alignment of the rods. Each region of orientated particles is a single liquid crystal and when rotated between crossed Nicol prisms shows extinction positions, but the bottom layer as a whole shows none. When a layer of spontaneously birefringent virus solution is examined with a lens or a microscope in polarised light, the regions of parallel orientation are seen as coloured areas of different hues. This indicates their different sizes and the fact that they are arranged at random, for the colour given depends on the path difference between the fast and slow ray in each, and this in turn depends on the thickness of the region and on the angle made by the direction of the rods with the plane of polarisation. The average size of the regions of parallel orientation depends on the concentration of the virus. It is largest in the most dilute and smallest in the most concentrated solutions.

BERNAL and FANKUCHEN (1939) have examined the liquid crystalline solutions by means of X-rays and confirmed the deductions made from the optical properties that they contain particles orientated parallel to one another. They have measured the distances between the particles in neutral solutions of different concentrations and revealed

a simple regularity of separation of particles not previously found in solutions. The distance between the particles in all liquid crystalline preparations is inversely proportional to the square root of the concentration (*see* Fig. 41). In other words, not only do the rod-like particles lie parallel but they are equidistant from one another, and are distributed so as to fill the available space as uniformly as possible.

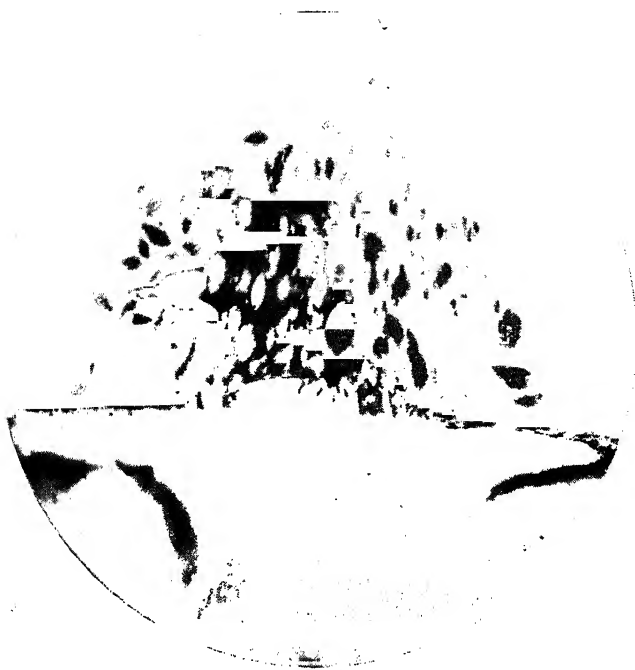


FIG. 32. — Photomicrograph, in polarised light, of the interface between top and bottom layers in a solution of tobacco mosaic virus allowed to settle in a cell 1 mm. deep. The photograph was taken after the fluid had stood for 6 hours, when birefringent spindle-shaped bodies were still being formed in the upper layer and settling out. The bottom layer when viewed is highly coloured. (BAWDEN, F. C. and PIRIE, N. W., 1937, *Proc. Roy. Soc. B.* 123, 274).

BAWDEN and PIRIE (1937*a*) observed that the degree of purification of tobacco mosaic virus preparations greatly affected the equilibrium concentrations between top and bottom layer solutions. Grossly impure preparations did not form layers at all, although birefringent jellies could be sedimented from them by high speed centrifugation. Less impure preparations layered if more concentrated than about 5%. In these conditions the difference between the concentration of the two layers in equilibrium was great, the top layer being about 4% and the bottom about 8%. On the other hand, when solutions were highly purified they separated into layers at concentrations as low as 1.6%. The difference in the solid contents of the two layers formed by such dilute preparations was small, often being less than one part in a hundred. In impure preparations, layering only when concentrated, appreciable differences could be detected between the serolog-

ical activity of the virus in the two layers, the impurities tending to concentrate in the upper layer. No differences at all could be detected in the activity or properties between the material in the upper and lower layers of solutions layering at concentrations around 1.6%. Moreover, the upper layers formed in this condition show an anisotropy of flow that persists for a long time after all flow movements have ceased. It is probable, however, that even in these preparations the top layer material still contains some slight impurity. With a perfectly pure virus preparation it is possible that the two layers could not occur together, for any condition affecting the orientation of virus particles would be expected to change the whole system from one state to the other.

Indeed, after successively formed bottom layer solutions have been diluted and the top layers removed, preparations are often obtained which change their state completely on the addition of a few percent of water or on changing the temperature a few degrees. But it would still be rash to assume that this is necessarily a proof of purity, for the study of this change of state is complicated by changes in other factors. As the purity increases there is a corresponding decrease in the difference between the specific gravities of the top and bottom layer fluids, so that the separation of the two layers may be extremely slow.

The properties of the two layers in equilibrium become more nearly the same as the purity of the preparation increases. This suggests that the equilibrium system is one in which the alteration of one variable causes a continuous transition from a distinct two phase system to a critical condition in which only one phase is possible. BERNAL and FANKUCHEN (1939) suggest that the most likely variable is the length of the virus particle. In the liquid crystalline solutions the virus particles are arranged parallel to each other. The possibility of maintaining this arrangement will depend on the ratio of the length of the particles to their distance apart, and beyond a certain critical ratio Brownian movement will make the arrangement unstable. Thus it can be seen that as the length of the particles increases, the concentration at which the solutions become liquid crystalline decreases. And it seems probable that the effects of purification lead to the formation of longer and longer particles, presumably by removing from the ends of the virus particles absorbed impurities which prevent them from becoming attached end-to-end. This theory satisfactorily explains the known data. The fractionation obtained by diluting bottom-layer fluid and then separating off the newly formed, more dilute bottom layer would result from the elimination of the shorter, contaminated particles in the upper-layer fluids. The fact that the serological titres of bottom-layer material separating from demonstrably impure preparations are higher than those of the upper layer although there is no corresponding increase in the infectivity, is further evidence suggesting that the units in the lower layer are larger aggregates than those in the upper. The results of three comparisons of the serological activity and infectivity of top- and bottom-layer materials separating from virus preparations made solely by precipitation methods are given in Table 14 (BAWDEN and PIRIE 1937a).

Table 14:

Activity of top and bottom layers separating from slightly impure preparations

Virus	Layer	Serological titre	Infectivity at various dilutions * Mean No. of lesions per half-leaf			
			10^{-4}	10^{-5}	10^{-6}	10^{-7}
Tobacco mosaic . .	Top	1/6,000,000	165	99	23	4
	Bottom	1/10,000,000	161	80	25	2.6
Aucuba mosaic . . .	Top	1/6,000,000	144	51	24	5
	Bottom	1/10,000,000	151	74	26	4
Enation mosaic . .	Top	1/3,000,000	75	27	6.5	0.16
	Bottom	1/6,000,000	72	27	7.5	0.5

In newly prepared solutions of tobacco mosaic virus, the limiting concentration at which solutions become liquid crystalline is about 1.6%. If allowed to stand for some months, solutions often become spontaneously birefringent at concentrations of 1% or less. This again probably results from a further aggregation of the virus particles, for if the solutions are diluted they show anisotropy of flow much more strongly than freshly prepared solutions of the same concentration. The limiting concentration depends to some extent on the temperature because of the variations in Brownian movement, and dilute solutions may be liquid crystalline at 0° C and lose this property at room temperature. The rate at which concentrated solutions separate into layers also depends on the temperature, being faster at 37° C than at 0° C. The initial conversion of an unstable virus solution into two phases proceeds faster if it is allowed to stand quite undisturbed, but once the process has started the actual separation into layers can be accelerated by centrifugation.

The birefringence of orientated preparations is sufficiently high to suggest that the particles themselves might be optically anisotropic. Nevertheless, LAUFFER (1938) has recently obtained results suggesting that the particles are optically isotropic, and that the optical effect results from the orientation of isotropic rods in an isotropic medium of different refractive index. LAUFFER measured the refractive index of a solution of tobacco mosaic virus of a known concentration and from this calculated the refractive index of the virus itself to be approximately 1.6. He then measured the anisotropy of flow of tobacco mosaic virus in solvents of different refractive indices, such as glycerol-water mixtures and aniline-glycerol-water mixtures, and found that it decreased with increasing refractive index of the solvent and was lost in a solvent with a refractive index of 1.55. As LAUFFER states that the activity of the virus was unaffected by the solvents used and the reduction of anisotropy of flow was not an effect of viscosity differences between the different fluids, this is strong evidence that the particles themselves have little or no intrinsic double refraction. Unfortunately, however, the point cannot be taken as proved. Until

* Dilution given as the weight of protein in grams per cc.

the virus has been examined in solvents of still higher refractive indices and shown to regain anisotropy of flow, the possibility of some properties (for example, dehydration) other than the refractive index of the solvent being responsible for the loss of anisotropy of flow cannot definitely be excluded.

Table 15:
Anisotropy of flow of 0.108% solutions of tobacco mosaic virus in solvents of different compositions

Composition of solvent in volume per cent			Refractive index of solvent	Amount of anisotropy of flow
Water	Glycerol	Aniline		
100	0	0	1.334	54
80	20	0	1.362	42
50	50	0	1.402	27
10	90	0	1.454	14
2	68	30	1.503	4.5
2	28	70	1.55	0

The virus solutions were made to flow through a capillary between crossed Nicol prisms. The light transmitted by the second prism was passed through a photoelectric cell to a galvanometer, and the anisotropy of flow is expressed as millimeters of galvanometer deflection (LAUFFER 1938).

When a beam of light is passed through virus solutions the light is scattered and they, like other colloidal solutions, show a Tyndall cone. LAUFFER (1938) has shown that the light scattering properties of liquid crystalline solutions of tobacco mosaic virus differ considerably from those of top-layer fluids. In the upper layer the scattered light is not depolarised appreciably, whereas in the bottom layer the scattered light is very largely depolarised, even when the incident light is polarised. The fact that the scattered light in the upper layer is not depolarised supports the view that the virus particles themselves have little intrinsic double refraction, for in suspensions of small isotropic particles the light scattered perpendicularly is completely polarised. The depolarisation of the light in the bottom-layer liquid will not result from the scattering by the virus particles themselves, but will be produced by the larger doubly refracting regions containing many orientated particles.

Only solutions of tobacco mosaic virus have yet been examined in any detail, but those of many other plant viruses show similar phenomena. A number of different strains of tobacco mosaic virus (aucuba mosaic, enation mosaic, masked tobacco mosaic and mild tobacco mosaic virus), cucumber viruses 3 and 4, three strains of potato virus "X" all show anisotropy of flow, and, when sufficiently purified and concentrated, give liquid crystalline solutions. At room temperature solutions of potato virus "X" will give a liquid crystalline layer until diluted to below 1.5%, when they show anisotropy of flow. Solutions of virus "X" are more viscous than those of tobacco mosaic virus at the same concentration, so that the separation of originally homogeneous solutions into layers takes place more slowly. Solutions of severe etch virus, potato virus "Y" and Hyoscyamus virus 3 also show ani-

sotropy of flow and give birefringent pellets when sedimented by high speed centrifugation, but these have not been obtained in sufficient quantities to determine whether they form liquid crystalline solutions. In the absence of any evidence to the contrary, it is reasonable to assume that all these viruses show these properties for the same reason as tobacco mosaic virus, but the possibility that some may possess intrinsic double refraction or be plates instead of rods cannot yet be excluded.

TAKAHASHI and RAWLINS (1933*b*) stated that some samples of clarified healthy tobacco sap also showed anisotropy of flow. Later workers have been unable to confirm this observation, even when concentrated solutions of normal plant proteins have been examined. The simplest explanation that can be offered for the results of TAKAHASHI and RAWLINS is that some of the samples of sap examined as virus-free actually contained virus, although the plants from which they were expressed were symptomless. In other words, the plants were infected with a masked strain of virus or had not been infected sufficiently long for obvious symptoms to develop. The optical properties of purified virus preparations differ so widely from those of the normal plant proteins that they supply an extremely convenient method of testing for the presence of viruses. However, the fact that healthy sap shows no anisotropy of flow cannot be taken as conclusive evidence that it contains no asymmetrical particles. Such particles may be in too small concentration to give the effect, their orientation by flow may be prevented by the presence of other substances, or their refractive index may be too close to that of the suspending medium. No anisotropy of flow can be detected in sap expressed from plants infected with viruses like potato virus "Y" until the virus has been greatly concentrated and freed from many impurities.

In general, the amount of anisotropy of flow shown by clarified sap depends upon its infectivity. However, as it is also affected by the amount of impurities present, it also depends on the methods used for clarification, being larger if the leaves are first frozen or the sap coagulated by heating before centrifugation. It is easily detected in extracts from frozen tobacco mosaic plants, and is small in similar extracts from plants infected with potato virus "X". The first is infective at dilutions of over 1/1,000,000, whereas the second loses infectivity at about 1/100,000, and LORING (1938*b*) states that the amount of anisotropy of flow shown by clarified juice from plants infected with virus "X" is of the same order as that of juice from tobacco mosaic plants diluted with about 25 parts of healthy sap. With other viruses yielding infective juices with smaller dilution end-points no anisotropy of flow has been detected in clarified sap. Evidence will be given later showing that the great asymmetry of particles in the purified virus preparations is partly an artifact produced by the purification methods. The fact that anisotropy of flow can be seen in infective sap, however, shows that rod-like particles are already present before the application of these methods.

Crystals and liquid crystals:—The name liquid crystal was first coined to describe liquids possessing the property of double refraction.

The essential difference between a true crystal and a liquid crystal, however, is not that one is a solid and the other a liquid, but is the degree of regularity with which the constituent units are arranged. Normally, crystallinity presupposes an indefinite repetition of identical units in three dimensional space, and, as an external indication of their internal regularity, true crystals are usually bounded by plane surfaces symmetrically arranged. In liquid crystals the regularity is incomplete. Between the perfect regularity of a true crystal and the completely random arrangement characteristic of amorphous materials there are many possible arrangements showing intermediate degrees of regularity. Some of these are known as liquid crystals. The only one which need be considered here is one that although recognised as theoretically possible had not previously been discovered. It is formed by the parallel orientation of particles at equal distances from one another, so that there is a two-dimensional regularity at right angles to the direction of the particles but no regularity of arrangement in the direction of their length. Such a degree of orderly arrangement is confined to liquids and wax-like solids. These solids are said to be paracrystalline or in the mesomorphic state.

Solid forms of tobacco mosaic virus which are birefringent can be obtained in a number of ways. When neutral solutions of the virus are centrifuged at high speeds birefringent gels are deposited. The solid content of the gels depends directly on the concentration of the fluid centrifuged, and varies between 10 and 35%. Similar gels are also formed when neutral solutions are concentrated by evaporation. The structure in the gels is essentially the same as that in the bottom layer solutions, *i.e.*, they are composed of regions of parallel orientation arranged at random. However, they can be completely orientated by rolling between two glass slides or by being sucked into capillary tubes, so that the whole preparation behaves as a single liquid crystal and shows extinction positions when rotated in a beam of polarised light.

When solutions of purified tobacco mosaic virus are allowed to dry, a birefringent skin forms at the surface, showing that the particles are orientated relative to the air-water interface. If some solution is introduced between a microscope slide and a coverglass, kept apart by two glass threads, and allowed to evaporate from one surface, well orientated solid preparations of the virus can be obtained. During the drying the virus solutions pass through a number of different conditions. As the solutions are concentrated their birefringence increases to a limiting value of about 0.001 (BERNAL and FANKUCHEN 1939). The first distinguishable layer to be formed at the surface is a wet gel containing about 50% of water and with a higher birefringence of 0.006. On further drying, this layer shrinks by about 50% and passes into a dry gel with birefringence 0.003, intermediate between that of the liquid and the wet gel. The dry gel cracks if further dried and splits into single pieces of approximate dimensions $1 \times 0.5 \times 0.5$ mm., which can be examined by X-rays as if they were ordinary crystals.

Orientated preparations of different concentrations give X-ray patterns showing some remarkable differences and similarities. The patterns can conveniently be considered in two parts: one of scattering at large angles, which corresponds to small spacings, and the other

of scattering at small angles, which corresponds to large spacings. For the large angle scattering there is little difference between the patterns given by virus at all concentrations from the dry gel to top-layer solutions orientated by flow through capillary tubes. As this pattern is independent of the distance the particles are apart, it can only result from regularities within the virus particles themselves and can be referred to as the intramolecular pattern. The pattern for the small angle scattering varies with concentration of the preparation, the more dilute the solutions the greater the spacings, and clearly results from the regular packing together of the virus particles. The virus preparations, therefore, in a sense are doubly crystalline. The individual particles themselves are built up from sub-units which are arranged perfectly regularly in three-dimensional space. And they could be regarded as individual crystals, although the application of the word crystal to an entity too small to be visible would be unusual. The visible aggregates of tobacco mosaic virus, however, lack this three-dimensional regularity. All the intermolecular reflections observed in X-ray photographs lie in a plane at right angles to the length of the particles, that is, they refer to the relative sideways positions of the particles in the aggregates. A careful search has failed to reveal intermolecular reflections in a direction perpendicular to the orientation axis. Hence, there is no evidence of more than a two dimensional regularity in the arrangement with which the particles pack together.

The most complete X-ray pattern is given by the dry gels. The reflections correspond to the first four planes of a two-dimensional hexagonal packing of side $15.2 \text{ m}\mu$ (BAWDEN and others 1936; BERNAL and FANKUCHEN 1939). The lines are sharp, indicating that the areas of regular packing are large compared with the size of the particles, and agree perfectly with those calculated for a hexagonal lattice. The patterns for wet gel and orientated fluids are less complete, but again correspond to a hexagonal packing. In the wet gel the spacings correspond to an intermolecular distance of $21 \text{ m}\mu$, and in the fluids the spacings increase with the dilution.

The intense satin-like sheen produced by the addition of acid, or of a quarter of a volume of saturated ammonium sulphate solution, to purified tobacco mosaic virus preparations suggests that the precipitated material may be in the crystalline state. The individual particles are needle-shaped and birefringent. They taper at both ends, show no obvious facets and resemble fibres as much as crystals. They average about 40μ long and 0.4μ thick, and are too small for a conclusive microscopic examination. STANLEY (1937*b*) and WYCKOFF and COREY (1936) concluded that they were true crystals, but later work has shown that they are more accurately described as paracrystals or mesomorphic fibres. There is no evidence that the virus particles are arranged in the needles with a higher degree of regularity than that demonstrated in the preparations described as wet gel. That is, there is a regularity of arrangement in the cross-section, but no evidence of any along the length.

If the precipitate produced by the addition of acid is sedimented in an ordinary centrifuge tube, a dense white precipitate results containing about 75% of water. On the other hand, if it is centrifuged

in the end of an L2 Chamberland filter candle, the acid precipitate is converted into a soft, translucent mass with a water content of about 50%, that is, similar to the water content of the wet gel. This translucent precipitate can be orientated completely by rolling between glass slides, when it becomes transparent and highly birefringent (BAWDEN and PIRIE 1937*d*). In this condition its optical properties and X-ray pattern are indistinguishable from those of wet gel. However, when it is soaked in water and shaken it returns to a suspension of needles with the characteristic sheen. It seems, then, that the particles in the needle-shaped precipitates are also packed with a perfect two-dimensional regularity at right angles to their length, but again there is no evidence of regular arrangement in the direction of their length. Their most probable internal arrangement is that shown in Fig. 33 (BERNAL and FANKUCHEN 1937).

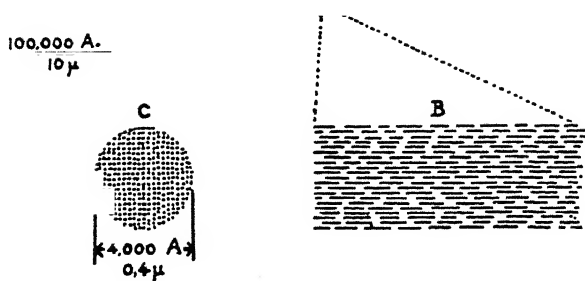


FIG. 33. — Diagrammatic representation of tobacco mosaic virus paracrystals. A: Typical paracrystal. B: Longitudinal section. C: Transverse section. Note regularity of arrangement in cross-section, but none in the direction of the length of the particles. (BERNAL, J. D. and FANKUCHEN, I., 1937, *Nature* 139, 923).

Attempts have been made to increase the size of the paracrystals by slow precipitation methods (BAWDEN and PIRIE 1937*a*). These failed, but suggested that the first step in the production of the paracrystals is the formation of a gel. If neutral solutions of the virus are kept quite still while the acidity or salt content is slowly increased, they gradually turn into limp jellies. When these are shaken or stirred they immediately break up to give a suspension of needles. The process of gel formation is most conveniently studied in mixtures containing 0.5–1.0% of the virus, 0.25–0.5% of glycine and 5% of neutralised ethyl formate. The pH is adjusted to about 5 and the fluid immediately placed into vessels in which it can be observed without being disturbed. As the ethyl formate undergoes hydrolysis, the pH is reduced and ultimately reaches the precipitation point 3.3. No change in the optical properties of such dilute solutions occurs, although the mixture turns to a fairly rigid jelly. If stirred it changes instantly to a suspension of needles with the typical sheen. It now behaves exactly like virus solutions to which acid has been added directly, and if allowed to stand undisturbed does not revert to the gel state. If more concentrated solutions are slowly acidified in this way they behave rather differently. The undisturbed gel breaks up

to give a mosaic of birefringent spindles disposed at random, resembling bottom-layer fluids. When stirred, however, this also breaks up to give a suspension of needles.

These phenomena all suggest that the visible precipitates of tobacco mosaic virus are more accurately described as fibrous than as crystalline, and that their regular appearance under the microscope is to be attributed to the more or less regular breaking up of a gel rather than to the wholly regular process of crystal formation. Other properties of the needles also serve to identify them as pieces of gel. On pressing, they readily fuse together, so emphasising their partially liquid nature. Also, if the precipitate obtained at pH 4.2 in salt-free solutions is centrifuged off, it produces an opaque, white mass. When this is covered with an N/10 salt solution and allowed to remain undisturbed, the upper layers gradually change to a transparent, highly birefringent,

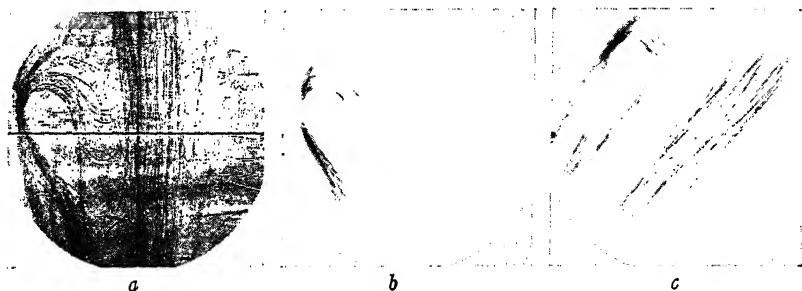


FIG. 34. — *a*. Photomicrograph of portion of mesomorphic fibres produced in the clarified sap from tobacco plants suffering from mosaic after it had stood at 1°C for several months. *b*. The same field as in *a*, photographed between crossed Nicol prisms. *c*. The same field between crossed Nicols after rotating the stage through 45°. $\times 60$. (BEST, R. J., 1937, *Nature* 139, 628).

jelly. If slightly agitated, the jelly breaks up to give rise to long birefringent fibres, presumably similar to those described by BEST (1937*b*) in purified virus preparations around this pH value. For if the suspensions are now shaken vigorously, the long fibres break up and the virus apparently goes into solution. By transmitted light such solutions are quite transparent, but by reflected light they show an intense sheen. They are highly viscous and slightly thixotropic; if allowed to stand undisturbed they turn into birefringent jellies but can be made to flow again if stirred or shaken.

BEST (1937*a*) has described the virus as settling out of clarified infective sap that has been kept for some months at 1°C in the form of long mesomorphic fibres. When undisturbed, the fibres were several centimetres long and fairly flexible, but when mounted under a cover-glass they usually broke into fragments 2–5 mm. long. In polarised light, these were birefringent with straight extinction (Fig. 34). Gentle agitation broke them up still further to the form of small needles and violent shaking reduced most of them to a state at which they were no longer visible. When again allowed to stand undisturbed the long fibres reappeared. BEST considers these fibres to be pure virus, but unless the sap became very acid or extra salt had been added this seems improbable. Purified preparations of tobacco mosaic virus do

not form such fibres even when stood in the cold for a year unless the pH falls to below 5 and the fluids contain a good deal of salt. It is possible that the long fibres described by BEST are insoluble complexes formed by the virus uniting with some protein breakdown product from the plant sap. Birefringent fibres which behave in a somewhat similar manner can be formed by adding some histones, protamines or nicotine to solutions of the purified virus.

Suspensions of paracrystals produced by precipitating the virus with acid or ammonium sulphate show anisotropy of flow at greater dilutions than neutral solutions. This again suggests that the constituent rods lie parallel. An increase in anisotropy of flow, however, is produced by the addition of acid or ammonium sulphate in quantities insufficient to precipitate the virus in the form of microscopically visible needles. This increase probably results from the end-to-end association of the particles to form still longer, and therefore more easily orientated, particles. LAUFFER (1938a) has determined the specific viscosity of tobacco mosaic virus solutions at various hydrogen ion concentrations and obtained further evidence for the two stages in the production of paracrystals. Between pH 7 and 5.5 the viscosity is almost constant, but it rises sharply between pH 5.5 and pH 4 indicating a great increase in the length to width ratio of the particles. Near the isoelectric point, where the virus is precipitated, the viscosity falls to a value approximately the same as that at pH 7. By contrast, the anisotropy of flow is almost unchanged between pH 5.5 and 3.3. The difference in the behaviour of anisotropy of flow and viscosity at the isoelectric point can possibly be explained by changes in the length to width ratios of the virus aggregates. According to LAUFFER, viscosity is a function of both the length and thickness of particles, whereas anisotropy of flow is determined chiefly by the length. Thus, if the first stage in the formation of visible aggregates is the formation of longer particles by a linear aggregation, both viscosity and anisotropy of flow would be increased. On the other hand, if the second stage is a side-to-side association of these long thin aggregates, viscosity would be greatly reduced while anisotropy of flow would be little affected.

From most points of view the distinctions between true crystals and solids in the liquid crystalline state are of little practical importance. But there are two reasons why it is of value to differentiate between the paracrystals of tobacco mosaic virus and true crystals. Firstly, it prevents the apparent crystallinity of virus preparations being advanced as evidence of their purity. Were they true crystals their formation would by no means necessarily show that the preparations were homogeneous, for a number of crystalline protein preparations (*e.g.* trypsin) at first thought to be homogeneous have later been shown to be heterogeneous. If they are regarded as pieces of jelly containing 50% of free water between the particles, however, this danger is avoided, for it is obvious that they can contain impurities and that the fact that repeated "recrystallisations" has no effect on activity cannot be taken as evidence of homogeneity. Secondly, the differentiation is important because it indicates that the virus particles as formed in the plant may be in a different physical state from those in the purified preparations.

No solid preparations of purified tobacco mosaic virus have yet been produced *in vitro* in which a three-dimensional regularity has been demonstrated. In the infected plants, however, true crystals occur in large numbers. For reasons discussed in Chapter 3, these crystals are unlikely to be pure virus, but that they are rich in virus is shown by their breaking up on the addition of acid into needle-shaped fibres,

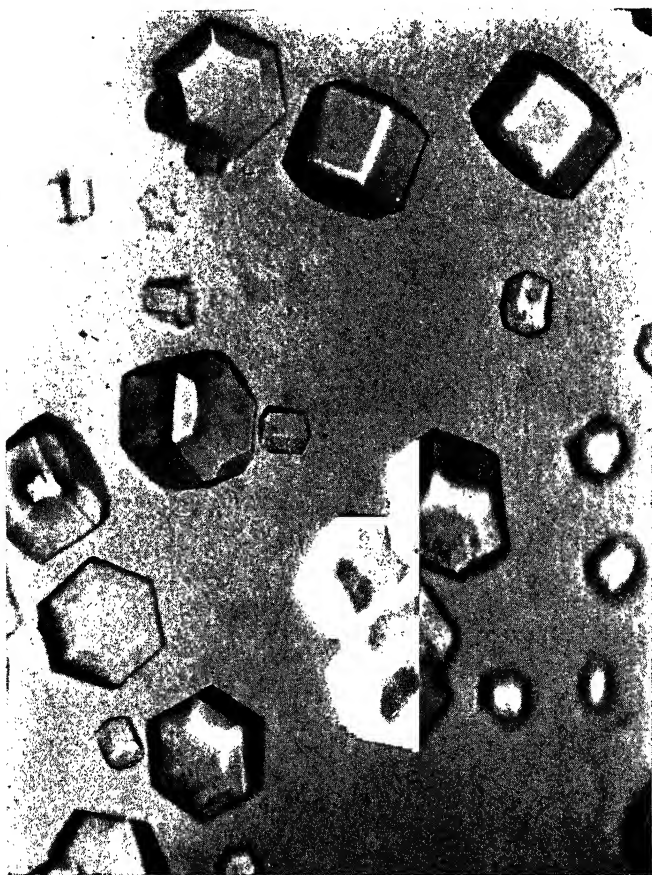


FIG. 35a. — Hexagonal birefringent prisms formed by a tobacco necrosis virus. — a, Photographed by transmitted light.

microscopically indistinguishable from those produced by the addition of acid to solutions of the purified virus. Their relation to the virus is further indicated by their constant association with infection, by their hexagonal habit and positive birefringence. That these possess a full three-dimensional regularity, and hence contain particles of identical length, is shown by the fact that they are bounded by plane surfaces symmetrically arranged. It is, of course, possible that in the plant the virus unites with a substance which has not been tested *in vitro* to form true crystals, or that in the plant a process of slow crystallisation goes on that has not been simulated *in vitro*. BERNAL (1938), however,

considers that the crystals are typical of less asymmetrical particles than those found in purified preparations. Hence, it is possible that, in spite of their great asymmetry *in vitro*, the virus particles as produced in the plant are more nearly spherical. The ready transition of the material in the crystals to the paracrystalline, needle-form suggests that these postulated primitive particles are already arranged in rows, and that the effect of acid is first to remove intercalary materials, so



FIG. 35*b*. — Hexagonal birefringent prisms formed by a tobacco necrosis virus. — *b*, Photographed between crossed Nicol prisms.

allowing them to aggregate into separate long rods, and to form the visible needle-shaped aggregates. KAUSCHE (1939) claims that the virus can pass from the needle form to hexagonal crystals *in vitro*. He precipitated virus from sap with ammonium sulphate and then added clarified infective sap to the virus-containing precipitate. KAUSCHE examined this mixture microscopically and stated that the hexagonal crystals developed out of the precipitate of needles. At Rothamsted we have been unable to confirm this. When such mixtures of sap and precipitated virus were made, the precipitated virus slowly went into solution, unless the concentration of ammonium sulphate was too great when it remained unaffected. The only crystals formed were those of calcium sulphate, produced from the calcium salts in the sap and the ammonium sulphate in the precipitated material.

Enation mosaic virus and aucuba mosaic virus behave in essentially the same manner as tobacco mosaic virus both in the plant and

in vitro. Cucumber viruses 3 and 4 also resemble tobacco mosaic virus *in vitro*, but no crystals have yet been seen in plants infected with these. Strains of potato virus "X" resemble tobacco mosaic virus in some ways, but differ strikingly in others. Dilute solutions show anisotropy of flow, concentrated solutions are liquid crystalline, and birefringent solid preparations in the form of gels can be obtained by sedimenting the virus from neutral solutions by high speed centrifugation, or by concentrating them by evaporation. But when virus "X" is precipitated with acid or ammonium sulphate it does not form needles with a characteristic sheen. Instead, the precipitate is flocculent and looks quite amorphous under the microscope. In sharp contrast to tobacco mosaic virus, neutral solutions of potato virus "X", at concentrations at which they show anisotropy of flow strongly, lose this property when the virus is completely precipitated. Similarly, if clupein is added to neutral solutions of potato virus "X" the resulting precipitates are amorphous and not paracrystalline. When acid or ammonium sulphate is added to solutions of virus "X" in insufficient quantities to cause precipitation, their behaviour approximates more closely to that of tobacco mosaic virus solutions. At about pH 5, or in about 10% saturated ammonium sulphate solution, the preparations develop a definite sheen and show increased anisotropy of flow. It seems, therefore, that in the first stages of precipitation the virus particles become attached end-to-end, as with tobacco mosaic virus, but when these particles are forced out of solution they come together in quite a random manner. Potato virus "Y" also seems to behave in the same manner as virus "X", but it has not yet been examined in such detail.

Such conflicting statements have been made about the behaviour of tobacco ringspot virus that its optical properties are uncertain. STANLEY and WYCKOFF (1937) first claimed the isolation of this virus in the form of a "high molecular weight crystalline protein", but no reasons were given for describing their product as crystalline. Later LAUFFER and STANLEY (1938) and STANLEY and LORING (1939) stated that preparations of this virus showed anisotropy of flow and that its particles were rod-like. In his latest paper on this virus, however, STANLEY (1939) appears to withdraw all his previous claims for any kind of crystallinity, for the virus is said to have spherical particles, solutions are said to be isotropic and when salted out the virus gives only amorphous precipitates.

The other viruses so far purified, those causing bushy stunt, tobacco necrosis and alfalfa mosaic, are alike in some respects but differ in others. Solutions of each, and the gels sedimented by high speed centrifugation, are isotropic. If the gels are examined in polarised light immediately after centrifugation, or after they have been stirred, they may be faintly birefringent, but this is merely a photoelastic effect and disappears when the forces producing strain are eliminated. Alfalfa mosaic virus is flocculated by acid but only amorphous precipitates have been produced either with acid or salts. The viruses causing bushy stunt and tobacco necrosis are soluble at their isoelectric points, and treatments with acid or salts do not appear to cause the particle to aggregate linearly. These viruses differ strikingly from the others

in their behaviour when precipitated with salt, for they separate as well-defined crystals.

The crystals formed by bushy stunt virus in many different preparations over several years have all been isotropic, belonging to the cubic system. The vast majority are rhombic dodecahedra (Fig. 23) but occasionally a few icositetrahedra or flat hexagons are formed. The X-ray pattern from the crystals shows spacings corresponding to a body-centred lattice, and it is highly probable that the particles are spheres. Bushy stunt virus also precipitates in the form of isotropic crystals when mixed with neutral solutions of clupein sulphate. From plants suffering from tobacco necrosis, viruses behaving in a variety of ways when precipitated with salts have been isolated (BAWDEN and PIRIE 1942). Solutions of all of them are isotropic, so that the particles are not grossly anisodimensional, although it would be unjustified to assume that they are spherical. When precipitated with ammonium sulphate, the suspensions give a definite sheen when stirred, and the precipitated material shows anisotropy of flow if shaken between cross polarisers. If precipitated slowly in the cold, some of these viruses and virus strains have formed well-defined crystals whereas others have not. These individual variations are described in Chapter 8. These tobacco necrosis viruses are the only ones so far isolated which have given true crystals that are birefringent. Fig. 35 shows hexagonal prisms with twenty facets formed by one tobacco necrosis virus; these are birefringent when viewed along one axis but not when viewed along the other. An alternative crystal form of this virus, hexagonal prisms with pointed ends and only eighteen facets, is pictured in Fig. 16.

INACTIVATION OF VIRUSES

Since JOHNSON (1927) pointed out that the resistance of different viruses to certain physical and chemical treatments varied widely, most workers who have described mechanically-transmitted viruses have given data on such properties as thermal inactivation point, longevity *in vitro* and resistance to alcohol as an aid to identification. It is customary to test such properties in crude, undiluted sap, and for viruses which produce similar symptoms but differ widely in stability the tests are of considerable value in diagnosis. For several reasons, however, such tests may give misleading information about the intrinsic stability of some viruses. The virus content, and the ratio of virus to normal host constituents, can vary considerably in different samples of infective sap; as loss of infectivity is a gradual process and can be conditioned by materials in the inoculum, such variations could obviously affect the results of tests. The thermal inactivation points of different viruses in sap range from 42° C to 92° C, and longevity *in vitro* from a few hours to years, but a large number of viruses lose infectivity after 10 minutes' heating around 55° C or after standing for a few days at room temperature. These are the conditions in which many plant proteins become denatured, and, as some viruses are readily absorbed on to other materials, it is to be expected that when these normal proteins precipitate they will carry with them some, or all, of such viruses. The pH of the sap and the presence or absence of oxidases are also factors that can influence inactivation. Tomato spotted wilt virus, for example, is inactivated in untouched sap in a few hours at room temperature. But if the sap is adjusted to pH 7, the longevity is considerably increased, and if a reducing substance is also added, or if oxygen is excluded, the virus will remain active for as long as 35 days (BEST 1937). Thus, this virus is not inactivated in sap because it is inherently unstable *in vitro*, but because the conditions of pH and oxidation-reduction potentials destroy infectivity. Similarly, potato virus "Y" remains infective *in vitro* for longer, and is less readily inactivated by acid, if it is freed from normal plant components. Because of the difficulties in interpreting the results obtained with infective sap, in this chapter the discussion will deal chiefly with viruses which have been studied after purification.

For studying inactivation most workers have used infectivity tests, and loss of infectivity has been taken as synonymous with destruction of the virus. Recent work has made it increasingly clear that this is not necessarily so, for three different types of reaction leading to loss of infectivity can now be distinguished. The first is readily reversible, and is better described as inhibition or neutralisation of infectivity than as inactivation. The second type is not readily reversible, but the loss of infectivity is not accompanied by any other great changes in the

virus preparations, which retain their serological activity and characteristic physical and chemical properties. The third type of reaction leading to loss of infectivity causes the denaturation or breakdown of the virus particles, with loss of all their characteristic properties. Some treatments cause loss of infectivity only or predominantly in one of the ways, but no absolutely clear-cut distinction can be drawn, for other treatments may act in more than one way, or act on one virus in one way and on a second in another.

Inhibitors of infectivity:—A number of substances have now been found which act as inhibitors of infectivity. Immediately they are added to virus preparations they cause a loss of infectivity, the loss being proportional to the amount added. Prolonged contact between these substances and the viruses usually has no further effect in decreasing infectivity, and if such non-infective mixtures are tested against virus antisera they precipitate in the same way as normal virus preparations. These inhibitors have no permanent effect on the viruses, for loss of infectivity occurs only when they are present in the inoculum, and if they are removed from the preparations the viruses are again fully infective. These two phenomena sharply distinguish inhibitors from substances which inactivate by destroying the viruses, for the loss of infectivity produced by the latter increases with period of contact and there is no recovery of activity when such substances are removed. Most work on inhibitors of infectivity has been done with tobacco mosaic virus, but the phenomenon is probably a general one, for some substances which inhibit tobacco mosaic virus have been tested on others and have reduced the infectivity of these also.

Some of these inhibitors, *e.g.*, *Phytolacca* juice (DUGGAR and ARMSTRONG 1925), trypsin (LOJKEV and VINSON 1931), and growth products from *Aerobacter aerogenes* and *Aspergillus niger* (JOHNSON and HOGGAN 1937) were first thought to destroy the virus. CALDWELL (1933), however, showed that mixtures of tobacco mosaic virus and trypsin could be largely reactivated by heating, so that the inactivation clearly was not a result of proteolysis. STANLEY (1934a) confirmed this. He also showed that the reduction in infectivity occurred immediately, that no further inactivation was produced by incubation, that it occurred at pH values at which trypsin is inactive as an enzyme, and that infectivity could be regained by diluting the virus-enzyme mixtures or by destroying the enzyme with heat or with digestion by other proteolytic enzymes. BAWDEN and PRIDE (1937a) also showed that the virus could be recovered, apparently with full activity, from non-infective mixtures by precipitation with salt or acid. These results show conclusively that tobacco mosaic virus is not destroyed by trypsin, but they do not indicate the reasons for the loss of infectivity. There seem to be two possibilities. One is that the enzyme has no direct effect on the virus but acts on the host plant to decrease susceptibility and so apparently reduce infectivity. The second is that the virus and enzyme combine to form a loose complex, which is non-infective but readily breaks down to liberate infective virus when the mixture is diluted. The difficulties and uncertainties of distinguishing between reduction in infectivity caused by substances acting on the virus and those acting on the host are discussed in Chapter 2.

STANLEY attempted to test the possibility of the formation of a complex by measuring the rates of diffusion of trypsin in the presence and absence of tobacco mosaic virus. He found no differences, and concluded that no complex was formed and that the effect was on the host plant. HILLS and VINSON (1938), on the other hand, got different results from similar experiments. They claim that the apparent size of tobacco mosaic virus is increased in the presence of trypsin, and that the diffusion rate of trypsin alone is one-third greater than when tobacco mosaic virus is present. With such conflicting evidence, definite conclusions on the mechanism of inhibition cannot be drawn.

In normal proteolysis there are believed to be three stages. First the enzyme and substrate unite, the substrate is then split and finally the enzyme is liberated. It is possible that with tobacco mosaic virus and trypsin only the first stage occurs. Other viruses, such as tomato bushy stunt, tobacco necrosis and potato "Y", give a similar result with trypsin. Mixing with trypsin causes an immediate reduction in infectivity, but no further loss is produced by incubation. By contrast, potato virus "X" (BAWDEN and PRIE 1936) and alfalfa mosaic virus (ROSS 1941) are hydrolysed by trypsin. When mixed with the enzyme, preparations of these viruses, like those of tobacco mosaic virus, suffer an immediate loss of infectivity, but if the mixtures are then incubated in conditions in which trypsin is proteolytically active there is a further progressive fall in infectivity. This second fall is accompanied by a corresponding loss of serological activity, and the incubated mixtures do not regain infectivity if the trypsin is removed. Other proteolytic enzymes do not have an inhibitory effect similar to that of trypsin. When mixed with pepsin, for example, there is no immediate fall of infectivity. Potato virus "X" is rapidly hydrolysed by incubation with pepsin, but tobacco mosaic, bushy stunt and tobacco necrosis viruses are not. Virus "X" is also destroyed by incubation with papain, although this, like pepsin, does not cause an immediate loss of infectivity when added. As potato virus "X" is hydrolysed by these three enzymes, it presumably unites with all three. Only trypsin, however, acts as an inhibitor of infectivity, and it seems that if its specific action is not on the host plant then it must unite with the viruses in a manner different from that of the other enzymes.

Some proteins which have no proteolytic activity can also act as inhibitors of infectivity; serum proteins, egg white, globin and trypsinogen all have pronounced effects. Specific antiserum, as described in Chapter 7, has a greater effect than unspecific sera. As specific sera flocculate the viruses, here there is obvious combination between the inhibitor and the viruses. But the effects are otherwise similar, for fully infective viruses can be regained from the non-infective mixtures of virus and antiserum by removing the antibodies and serum proteins. Plant and animal extracts of various kinds also provide strong inhibitors. BLACK (1939) has shown that viruses can be reversibly inactivated by extracts from crushed insects and he suggests that the inhibitor is a protein with low molecular weight. JOHNSON (1941) has reactivated preparations of tobacco mosaic virus made non-infective by the addition of milk, *Phytolacca* juice, growth products from *Aerobacter aerogenes*, tannin, and citrus fruit extracts, by allowing the

added products to diffuse away from the virus through agar. The presence of inhibitors in so many commonly occurring substances emphasises the danger of assuming that negative results in infectivity tests necessarily imply absence or destruction of virus. It also suggests a possible explanation for the failure of some viruses to be transmitted mechanically. For example, no strawberry viruses have been transmitted by inoculation; this may be because of some intrinsic property of the viruses themselves, but extracts of strawberry leaves are rich in tannin and if added to preparations of tobacco mosaic virus render them non-infective. Thus, even if the strawberry viruses were of the same type as tobacco mosaic virus, usually regarded as the easiest of all to transmit mechanically, it is unlikely that inoculation methods would be successful. Unless inhibitors from plants can act specifically on particular viruses, however, it is improbable that they account for most failures of mechanical transmission. For of pairs of viruses affecting the same host, *e.g.*, potato "X" and leaf roll or mosaic and yellows of sugar beet, one is often readily transmitted by inoculation of sap whereas the other is not.

Inactivation without loss of serological activity:— Infectivity is the most sensitive property of virus particles and is often lost completely by changes too slight to affect other properties significantly. As a result, non-infective preparations can be obtained with all the serological and physical properties characteristic of normal virus preparations. The ease with which the separation of infectivity from other properties can be made varies with different viruses; in this section only those treatments that work readily with all the viruses to which they have been applied are described. Other treatments, described in the next section, readily produce a similar separation with some viruses but not with others.

The five treatments most generally useful for destroying infectivity without affecting serological activity are treatment with formaldehyde, nitrous acid or hydrogen peroxide and irradiation with ultra-violet light or X-rays. The non-infective preparations of tobacco mosaic virus and potato virus "X" show anisotropy of flow to the same extent as infective preparations of the same concentration, and, if sufficiently concentrated, form liquid crystalline solutions. When precipitated by acid or salts, such preparations of tobacco mosaic virus form paracrystalline needles indistinguishable from those of active virus. Similarly, preparations of bushy stunt virus inactivated by these methods crystallise well, and in the usual dodecahedra, when precipitated with ammonium sulphate (Fig. 36). The loss of infectivity is proportional to the length of treatment, or to the strength of the reagents used. By suitable treatments, therefore, preparations can be got with varying degrees of activity, but precipitating with antisera and salts in precisely the same manner. Attempts to fractionate such mixtures of infective and non-infective bushy stunt virus by successive crystallisations have failed, for each crystalline fraction possesses the same infectivity. Also, as the sedimentation constants of the virus are unaffected by this type of inactivation, no fractionation is to be expected from high speed centrifugation. Hence it is obvious that the

physical uniformity of virus preparations giving high serological titres cannot be taken as proof that they are homogeneous and consist solely of active virus.

In using nitrous acid or hydrogen peroxide as inactivating agents, care must be taken to avoid an excess of the reagents, for this can cause

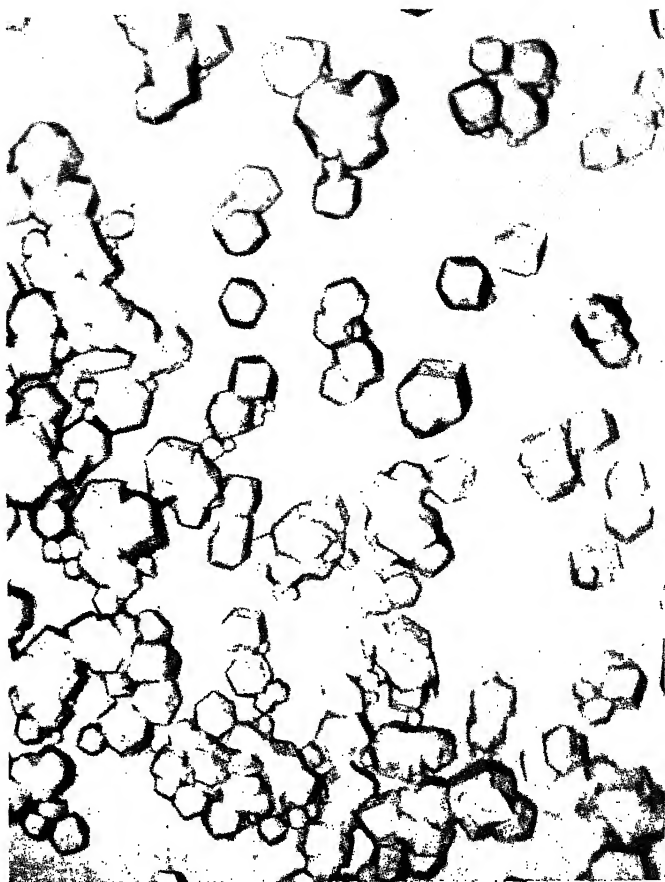


FIG. 36. — Crystals of tomato bushy stunt virus inactivated by irradiation with ultra-violet light. The crystalline material is still serologically active. $\times 300$. (BAWDEN, F. C. and PIRIE, N. W., 1938, *Brit. J. exp. Path.* 19, 251).

denaturation and loss of serological activity. The concentration necessary for this varies with different viruses. For example, tobacco mosaic virus is not denatured by 5% H_2O_2 acting for 5 hours, whereas potato virus "X" is denatured by concentrations of over 1%. Loss of infectivity without affecting the serological reactions of potato virus "X" occurs at concentrations between 0.2% and 1%. Excess of formaldehyde does not appear to cause denaturation. Nor do long periods of irradiation with X-rays or ultra-violet, provided that the solutions irradiated are kept cold. The results of irradiating 0.2% solutions of virus "X" are shown in Table 16.

Table 16:

The effect of X-rays and ultra-violet radiation on solutions of potato virus "X".

Radiation	Time	Serological titre	Infectivity. Mean number of lesions per leaf *
X-rays	100 mins.	$\frac{1}{2 \times 10^5}$	22
	360 mins.	$\frac{1}{2 \times 10^5}$	0
Ultra-violet	3.3 mins.	$\frac{1}{3 \times 10^5}$	40
	10 mins.	$\frac{1}{3 \times 10^5}$	1
	30 mins.	$\frac{1}{2 \times 10^5}$	0
Control	Not irradiated.	$\frac{1}{3 \times 10^5}$	342

In the ultra-violet test a layer 1 mm. thick, under a quartz plate, was irradiated at a distance of 8 cm. from a 2 amp. lamp. In the X-ray test, the solution was irradiated at 8 cm. from a copper anticathode of an X-ray tube run at 30 K.V. and 20 m.a. The wavelengths found by HOLLANDER and DUGGAR (1936) to be most effective in causing loss of infectivity are those at which purified preparations of tobacco mosaic virus have since been found to absorb most strongly. Wavelengths above 300 $m\mu$ have little effect, around 260 $m\mu$ where the purines of the nucleic acid absorb strongly there is an increase in efficiency, and lower wavelengths at which there is general absorption by the protein constituents of the virus are still more efficient. Inactivation by irradiation occurs over a wide pH range, but MARSHAK and TAKAHASHI (1942) find that the effect of X-rays on tobacco mosaic virus is greater at pH 2.2 than on the alkaline side of the isoelectric point.

The nature of the reactions responsible for inactivation of this type are still uncertain, but it is likely that they consist of minor changes in groupings or side-chains within the virus particles. Serological activity and ability to crystallise are much more closely linked with one another than with infectivity, and disruption of the particles, or at least some large structural change, is necessary to destroy these properties. Sufficient differences have been detected between active preparations and those inactivated by some of these treatments to show that the inactive proteins have undergone changes. STANLEY (1936c) has shown that the amino-nitrogen content of preparations treated with hydrogen peroxide or formaldehyde is lower than that of control preparations, and that, as would be expected, those treated with nitrous acid contain almost no amino-nitrogen. Solutions of the chemically treated, inactive material are rather more opalescent than untreated solutions, and the proteins are more easily denatured. The sedimentation constant of tobacco mosaic virus preparations is not significantly affected by these

* Concentration of protein in inoculum 10^{-4} gm. per cc.

treatments, but the boundary becomes more diffuse, especially after treatment with nitrous acid, showing that heterogeneity has been increased, although the large particles have not been disrupted (WYCKOFF, BISCOE and STANLEY 1937). According to STANLEY (1936c) the isoelectric point is unaffected by irradiation with ultra-violet light, but shifts to the acid side after treatment with formaldehyde or nitrous acid.

ROSS and STANLEY (1938) find that the loss of infectivity produced by treating preparations of tobacco mosaic virus with formaldehyde is in part reversible, for by dialysing at pH 3 some of the infectivity can be regained. They find that in the process of losing and regaining infectivity the proteins undergo demonstrable changes. By treating 2% solutions of purified virus with 2% formaldehyde in phosphate buffer at pH 7 for different lengths of time, they obtained preparations giving approximately 10%, 1% and 0.1% of their original infectivity. After 3 days dialysing at pH 3 the infectivity of these fluids increased to approximately 20%, 10% and 1% respectively of their original. They further found that some preparations which immediately after treatment with formaldehyde gave no lesions on *Nicotiana glutinosa* at 10⁻³ gms. per cc., produced lesions at this concentration after dialysis at pH 3. The increase in infectivity produced by the dialysis is strictly limited. Preparations having lost 99.9% were reactivated to 99%, and those having lost 99% were reactivated to 90%, of their original activity, but the former could not be reactivated to 90% by two treatments. This suggests that two reactions are concerned in the inactivation, only one of which is reversible. ROSS and STANLEY state that the reduction in infectivity after treating with formaldehyde is accompanied by a reduction in amino-groups and in groups reacting with Folin's reagent, and that the reactivation by dialysis produces a corresponding increase in these groups. They suggest that the groups reacting with Folin's reagent are the indole nuclei of tryptophane, and that reversible changes in these and in the amino groups are responsible for losing and regaining infectivity.

Although infectivity is the most sensitive indicator of changes in the virus particles, not all changes seem to destroy it. SCHRAMM and MÜLLER (1940) found that the amino groups of tobacco mosaic virus could be completely covered with acetyl or phenylureido groups without decreasing infectivity. Prolonged acetylation, however, did inactivate, which they suggested was because of phenolic groups becoming affected. MILLER and STANLEY (1941) could not get complete coverage without loss of infectivity, but they state that about 70% of the amino groups and 30% of the phenol plus indole groups can be covered without any inactivation. Similarly, ANSON and STANLEY (1941) were able to oxidise the sulphydryl groups of tobacco mosaic virus by means of iodine without reducing infectivity. If the treatment was sufficient to change the tyrosine to di-iodotyrosine the virus was inactivated and denatured.

Denaturation:— In general the treatments which are most effective in destroying all the characteristic properties are heating and ageing, and the addition of strong oxidising agents, protein precipitants or

reagents which cause wide changes in the hydrogen ion concentration. Individual viruses vary widely in their resistance to these treatments, and one virus may be more resistant than another to one treatment but more susceptible to others. Only a few viruses have been studied in any detail, but even so it is clear that viruses not only vary in susceptibility but also in the manner in which they break down. It is also clear that there is considerable interaction between the treatments causing denaturation and a term such as thermal inactivation point is largely meaningless unless the conditions under which the viruses are heated are clearly defined. For example, if tobacco mosaic virus is heated at pH 7 it is inactivated by 10 minutes at 75° C, whereas at pH 5.5 it is not completely inactive after 10 minutes at 90° C. Such differences can be equally well regarded as either a reduction in the thermal inactivation point by alkali or an increase in the alkaline inactivation by heat. The distinctions drawn between causes of denaturation in this section are, therefore, mainly arbitrary.

Heat and ageing: — Work on thermal inactivation has mainly been restricted to determining the minimum temperature at which ten minutes causes loss of infectivity. With most viruses, different workers have agreed within narrow limits as to the thermal inactivation point in expressed sap. With tobacco necrosis and tomato bushy stunt viruses, however, different workers have given widely different figures for the thermal inactivation points. The reasons for these discrepancies have become clear from recent work on the rate of inactivation at different temperatures and on the correlation between loss of infectivity and denaturation (PRICE 1938, 1940; LAUFFER and PRICE 1940; BAWDEN 1941; BAWDEN and PIRIE 1942*a*). With viruses such as potato "X" and tobacco mosaic, loss of infectivity on heating is closely related to denaturation. As with other examples of protein denaturation, the temperature coefficient, that is, the ratio between the rates of inactivation at temperatures separated by 10° C, is large. The fact that different workers agree so closely about the thermal inactivation point of these viruses can be explained on this fact. In the critical range, the increase in the rate of inactivation, with relatively small increases in temperature, is so great that variations in other factors, such as the virus content of sap or the susceptibility of the test plants, are unimportant. With tomato bushy stunt and tobacco necrosis viruses, on the other hand, the Q_{10} for thermal inactivation is small, for the loss of infectivity is not necessarily associated with denaturation. The range over which infectivity is lost fairly rapidly is wide, and increase in temperature is only a little more important than other factors in determining the precise inactivation point in any test.

The differences in the behaviour of the two kinds of viruses when heated for 10 minutes at various temperatures are shown in Table 17, the results of tests in which potato virus "X" and tomato bushy stunt virus were heated at pH 6. It will be seen that loss of infectivity is slight with potato virus "X" at 59° C, but complete at 9 degrees higher, whereas with tomato bushy stunt virus there is a range of over 30° C in which heating for ten minutes causes loss of infectivity. It will also be seen that loss of infectivity with potato virus "X" is

correlated with a fall in serological titre, whereas with bushy stunt virus there is no reduction in serological titre unless solutions are heated to above 80° C. The loss of serological activity with both viruses occurs over a range of only a few degrees, indicating a large Q_{10} for this phenomenon and that it is closely linked with denaturation.

Table 17:

Effect of heating potato virus "X" and tomato bushy stunt viruses for ten minutes at various temperatures and pH 6.

Potato virus "X"			Bushy stunt virus		
Temperature	Infectivity	Serological titre	Temperature	Infectivity	Serological titre
Unheated	108	1/256	Unheated	153	1/500
59° C	94	1/256	50° C	79	1/500
62° C	29	1/128	60° C	20	1/500
65° C	2.5	1/8	70° C	9	1/500
68° C	0	No ppt.	80° C	2	1/500
			85° C	0	No ppt.

There are similar differences between the behaviour of tobacco mosaic and tobacco necrosis viruses when heated for 10 minutes at various temperatures and pH 6. The serological activity of both is affected only at about 90° C, and the infectivity of tobacco mosaic virus is reduced only a little more than its serological activity. By contrast, tobacco necrosis viruses, which may not be completely non-infective after 10 minutes at 80° C, have their infectivity considerably reduced after 10 minutes at 50° C. Preparations of tomato bushy stunt and tobacco necrosis viruses with no demonstrable infectivity can be produced by heating for longer periods at temperatures well below the thermal inactivation points. A day at 50° C, a few hours at 60° C or 1 hour at 70° C give non-infective preparations, which crystallise well and are indistinguishable from infective preparations in their serological and physical properties. Such complete separation of infectivity from serological activity cannot be got with potato virus "X" and tobacco mosaic virus, although there is sometimes slight separation of the two properties. Potato virus "X" preparations at pH 6, when heated for 16 hours at 50° C, for example, may lose three-quarters of their infectivity but only one-quarter of their serological activity, and the loss of infectivity with preparations of tobacco mosaic virus is usually greater than would be expected from measurements on the amount of denatured protein produced. Thus it is clear that with all the viruses so far studied heat produces a series of reactions and that infectivity is lost because of changes early in the series. In viruses like potato "X" and tobacco mosaic these first changes are rapidly followed by others leading to the complete disruption of the particles, whereas with bushy stunt and tobacco necrosis viruses loss of infectivity apparently occurs because of intramolecular changes too slight to affect the stability of the particles. When heated sufficiently to cause denaturation the two kinds of viruses again behave differently, for the denaturation of tobacco mosaic virus and potato virus "X"

results in the separation of nucleic acid from the protein, whereas denaturation of bushy stunt and tobacco necrosis viruses does not. Not all the original antigens of tobacco mosaic and bushy stunt viruses are destroyed by denaturation. If rabbits are injected with the coagulum of heat-denatured viruses, they produce weak antisera that flocculate normal virus solutions. When heating is a little less than that needed to produce a coagulum, bushy stunt virus preparations become opalescent and material can be sedimented by centrifugation at 8,000 r.p.m. This material can be resuspended in saline and flocculated specifically by virus antiserum. The amount of heating needed to cause the formation of this easily sedimentable material, or to cause the separation of a coagulum, depends on the pH. In pH 4 buffer, solutions become opalescent after 10 minutes at 60° C and coagulate at slightly higher temperatures, whereas at pH 6 10 minutes at 80° C is needed (BAWDEN and KLECZKOWSKI 1942).

The same differences are found between the behaviour of viruses when inactivated by ageing *in vitro* as when inactivated by heat. Tobacco mosaic virus is so stable at room temperature that there is no information about its behaviour, but less stable viruses which lose infectivity and serological activity at about the same rate when heated, such as potato viruses "X" and "Y", also lose these properties at about the same rate on standing at room temperature. Infectivity is lost a little more rapidly than serological activity so that non-infective preparations can sometimes be obtained which react with antisera, but they give only low titres. Within a few weeks, purified preparations of potato "X" are non-infective, fail to react with antiserum or to show anisotropy of flow. By contrast, extracts from plants infected with bushy stunt or tobacco necrosis viruses, also become non-infective in a few weeks or months, but these retain their full serological activity. From such sap, non-infective crystalline nucleoproteins can be isolated that are indistinguishable in their physical, chemical and serological properties so far tested from normal virus preparations. This type of inactivation seems to occur more rapidly in sap than in purified preparations. It is likely that animal viruses will also be found to be of two types in their inactivation with heat and ageing. For differences of the type found between potato "X" and tobacco mosaic on the one hand, and tomato bushy stunt and tobacco necrosis on the other, would explain why heating or ageing produce successful vaccines with some animal viruses but not with others.

Drying and freezing:—Leaves and sap from plants infected with tobacco mosaic viruses are infectious after drying, a characteristic feature that has been widely used to separate them from other viruses. Similarly, drying has less effect on purified preparations of these viruses than on others. The material obtained by drying tobacco mosaic virus solutions in air or *in vacuo* over P₂O₅ readily dissolves in water or dilute salt solutions. The infectivity of solutions of dried virus is about one-half to one-third that of the original material, and there is a corresponding reduction in the serological titre and ability to show anisotropy of flow. If solutions are dried a second time, there is a further reduction in these three characters. After seven successive dryings

over P_2O_5 the activity is reduced to about 1% of the original. The inactivated part of a dried preparation behaves like many other impurities and inhibits the formation of a liquid crystalline layer. BAWDEN and PIRIE (1937a) found that the solid obtained by drying a 1.8% birefringent solution dissolved to give a solution giving only a trace of a liquid crystalline layer at 5.4% and none at 4%. Material dried more than once did not give a liquid crystalline solution at any concentration, unless stored for months when they became highly viscous and feebly birefringent. Solutions of dried material are difficult to fractionate. The inactive virus sediments in the high speed centrifuge at about the same rate as active virus and it precipitates with acid and salts in the same conditions. Unlike the active virus, however, it can be hydrolysed by trypsin, though only much more slowly than after denaturation by heat, and by incubating the dried preparations with trypsin, virus can be recovered with full infectivity, serological activity and optical properties.

No free nucleic acid can be detected in solutions of dried preparations of tobacco mosaic viruses, so it seems that the inactivated material is still a nucleoprotein. How inactivation occurs is unknown, but it may simply be because of mechanical damage as the particles pack tightly on the removal of water. This is suggested by the fact that drying in two stages, during the first of which the particles are completely orientated, does less damage than rapid drying in one stage. For example, if the water content is first reduced to 15-20% by slow drying over a mixture of Na_2SO_4 and $Na_2SO_4 \cdot 10 H_2O$, which itself has no inactivating effect, the removal of the remaining water by drying over P_2O_5 causes much less loss of infectivity and serological activity than if the original solution had been dried directly over P_2O_5 .

Other viruses behave differently when dried. Some, such as bushy stunt, tobacco ringspot and potato "Y", are inactivated and denatured under all the conditions in which they have been dried, whereas the behaviour of potato "X" and tobacco necrosis viruses is largely determined by the conditions of drying. The sap and leaves of plants infected with potato virus "X" lose infectivity when dried, and solutions of the purified virus also usually give much insoluble material. When partially dried over sodium sulphate, a birefringent film is formed which dissolves to give a solution identical with the original preparation. When completely dried over P_2O_5 , the film is isotropic and when taken up in water is less active and shows less anisotropy of flow than the undried virus. The exact loss varies with the pH of the solutions: in pH 6 buffer solutions the infectivity and serological activity are reduced to about 50% whereas at pH 4 and pH 8 they are reduced to about 10%. The effects of drying differ from those on tobacco mosaic virus. The inactivation of virus "X" seems to be accompanied by the breakdown of the particles and the separation of nucleic acid from protein. From the partially inactivated solutions, fully active virus can be sedimented by high speed centrifugation. In contrast to potato virus "X", drying does not inactivate tobacco necrosis viruses in crude sap but does in purified preparations. With these viruses drying may also produce non-infective material that reacts with virus antiserum. The activity of crude sap is not appreciably affected by drying and

re-resolution, but purified preparations dried unfrozen have their serological activity reduced to about one-half and their infectivity to about one-hundredth. When dried unfrozen, the material dissolves readily, but when dried frozen it is insoluble and inactive.

In general, freezing leaves or sap from infected plants has little or no effect on their infectivity. It facilitates the clarification of sap from tobacco and other solanaceous plants, by causing the denaturation of normal plant proteins, and is widely used as a first step in purification. With viruses such as tobacco etch and alfalfa mosaic there is loss of infectivity, but this is probably due to loss of virus on the coagulum of host protein rather than to destruction by freezing. After purification, however, some viruses are readily inactivated by freezing. This phenomenon was first described by BAWDEN and PIRIE (1938*b*), who found that purified preparations of bushy stunt virus were denatured by freezing in conditions that have no effect on the activity of potato virus "X" and tobacco mosaic virus. STANLEY (1940) found that bushy stunt virus was not inactivated by the freezing of infected leaves, and BAWDEN and PIRIE (1942*a*) found that the amount of inactivation is determined by the condition of freezing. The rate of inactivation is increased by increases in the concentration of the virus in the solutions frozen, by the addition of acid and by increases in the length of time the fluids are held frozen. The virus is protected against the inactivating effects of freezing by the presence of glucose, proteins, salts and other substances. The protection afforded by different salts depends on their salt:ice:water eutectic temperature. Those with a high eutectic temperature do not protect if freezing is carried out at a lower temperature. The concentration of virus in sap is so small and the *pH* is sufficiently far from the isoelectric point to account for the failure of freezing to inactivate. Added to this, the salts, sugars and colloids in the sap supplement the other protective effects. If infective sap is dialysed and brought to *pH* 3, then freezing causes inactivation. In general, loss of infectivity in frozen samples of bushy stunt virus is accompanied by a corresponding loss of serological activity and by the precipitation of denatured protein. However, inactivation by freezing, as by heating, is a complex process and loss of infectivity is produced by the earliest changes, so that frozen samples are sometimes less infective than would be expected from their serological titres. Occasionally, completely non-infective preparations with full serological activity can be prepared by freezing 0.2% virus solutions at *pH* 4.25 for 2 hours at -10°C .

Tobacco ringspot virus behaves in much the same way as bushy stunt virus when frozen (STANLEY 1939). When purified preparations are frozen and thawed, they are inactivated and 75% of the original material separates as a precipitate. This inactivation is also prevented by the presence of salts and other materials from plant sap. A rather different effect has been described with purified preparations of tobacco necrosis viruses (PIRIE and others 1938). After freezing, solutions lose their characteristic slight opalescence; there is loss of infectivity and a reduction in the precipitability with antiserum, especially in the region of antigen excess. Whether freezing in other conditions would cause changes more comparable with those with bushy stunt and tobacco

ringspot viruses has not been determined. It is probable that other viruses now thought to be unaffected by freezing and thawing would also be denatured if frozen under different conditions. Preparations of tobacco mosaic virus, for example, are unaffected by repeated freezings and thawings at the usual pH values, but freezing at pH 3 causes loss of infectivity and serological activity.

Hydrogen ion concentration:—The viruses so far studied differ more in their resistance to acid than to alkali. Potato virus "Y" is inactivated by pH values below 4.5, potato "X" and tobacco ringspot

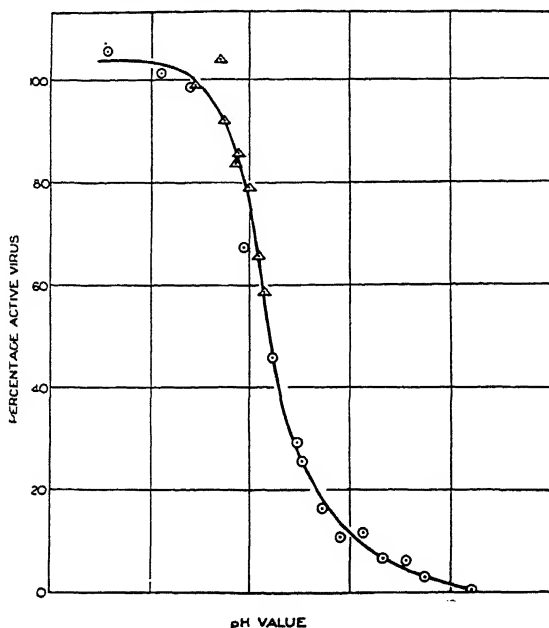


FIG. 37. — Showing the percentages of tobacco mosaic virus remaining active after 12 hours' storage at various pH values. Triangles represent values obtained with purified virus. Circles represent values obtained with clarified infective sap. (BEST, R. J., 1936, *Austral. J. exp. Biol. Med. Sci.* 14, 323).

below pH 4.0, whereas tobacco mosaic and bushy stunt viruses are stable at values down to pH 1.5. With all the viruses loss of infectivity produced by acid appears to be closely correlated with denaturation and loss of serological activity, but no careful comparisons have been made. Sedimentation studies with bushy stunt virus show that the extent to which the virus is broken down depends on the pH and time of exposure. After 30 minutes at pH 1.3 two components are produced with sedimentation constants only a little lower than that of the virus. After 48 hours, the preparations appear to contain only one component with a sedimentation constant less than one-half that of the virus (McFARLANE and KEKWICK 1938). At pH 1 the originally opalescent virus solutions rapidly become water-clear. Nothing can be sedimented from such solutions by centrifuging at 16,000 r.p.m., but if they are neutralised denatured protein precipitates spontaneously.

There is general agreement that tobacco mosaic virus, in spite of its greater resistance to most other treatments, is no more resistant to alkali than other viruses that have been studied, but different workers have given different figures for the actual pH necessary to cause inactivation. BEST (1936) found that inactivation set in at about pH 7.8, and that the amount of inactivation in 12 hours at room temperature was progressively larger with increasing pH , until at pH 10.2 only 0.5% of the infectivity remained. Between pH 8.0 and 8.9, giving 20% and 90% inactivation respectively, the ratio of the concentration of hydrogen ions to remaining active virus was a constant (Fig. 37). BEST concluded that inactivation was produced by neutralisation of acidic groups, and with the discovery that the virus was a nucleoprotein he later (1937b) suggested that these might be the nucleic acid fractions. BAWDEN and PIRIE (1940a) found their preparation to be more resistant to alkali and exposure at pH 10 or so was necessary to cause appreciable inactivation. It is possible that these discrepancies arise because of the use of different strains or because of different treatments to which the preparations had been previously exposed.

The results of exposing a purified preparation of tobacco mosaic virus, made by precipitation methods, to various degrees of alkali are shown in Table 18. It is clear that the action is complex, for the variations in infectivity and serological titre are not correlated. There is no fall in activity below pH 10.5, and with this treatment there is obviously loss of infectivity without a corresponding fall in serological activity. Similarly, at pH 11 there is still some serological activity, though the preparations are not infective.

Table 18:
The effects of alkali on tobacco mosaic virus

pH	Time	Serological titre	Average number of lesions per leaf at	
11.0	24 hr.	1/40,000	0	0
10.5	24 hr.	1/3,000,000	12	2
9.3	24 hr.	1/4,000,000	106	21
8.5	24 hr.	1/4,000,000	74	9
8.5	5 min.	1/4,000,000	65	11
Control		1/4,000,000	73	10

Exposure at pH 9.3 shows an effect frequently obtained in such tests, an apparent increase in infectivity with no change in the serological titre. This activation can be regarded as a partial reversal of the fall in infectivity produced by vigorous chemical purification, which is believed to be caused by the linear aggregation of virus particles, and the simplest explanation is that gentle treatment with alkali disaggregates the long rods. Such an effect might be more than sufficient to mask any small amount of inactivation occurring simultaneously. Thus alkali can have at least three successive effects on purified preparations of tobacco mosaic virus. First, there is a disaggregation into

smaller active particles, secondly a change within the particles, making them non-infective without destroying their structure or serological properties, and thirdly a disruption of the particles leading to loss of all characteristic properties. The course of the third stage is also influenced by the temperature, for at low temperatures the denatured products remain soluble whereas at high temperatures they precipitate. The centrifugal studies of ERIKSSON-QUENSEL and SVEDBERG (1936) and WYCKOFF (1937) suggest that the third stage is also complex, for the extent to which the virus is split into products of smaller molecular weight is increased by increased exposure to alkali.

The pH range over which bushy stunt and tobacco necrosis viruses lose infectivity without undergoing denaturation is wider than that of tobacco mosaic virus, and, as with heating, it is correspondingly easier to produce non-infective preparations that are fully active serologically. Exposure of these viruses to pH 9 or so causes fairly rapid loss of infectivity, but serological activity and ability to crystallise are not affected until the pH is raised to 10 or higher. Even after denaturation sets in and preparations of bushy stunt virus lose their molecular homogeneity, they can still react with virus antiserum. For example, after 2 hours' exposure at 18°C to pH 11, a part of the preparation precipitates on neutralisation, but the remaining soluble material still reacts specifically with virus antiserum. The behaviour of virus "X" has not been studied in detail, but there does not seem to be a wide pH range in which loss of infectivity occurs without denaturation, for LORING (1938*b*) states that at pH 10 and above, where infectivity was lost, the homogeneity of the preparations was also destroyed.

Miscellaneous treatments:— Various substances known to act as protein precipitants or denaturants have been found to inactivate viruses. Of these, the most studied is urea. BAWDEN and PIRIE (1937*a*) found that three strains of tobacco mosaic virus were denatured and lost their liquid crystallinity after some hours' exposure to saturated urea, and MEHL (1938) also described loss of anisotropy of flow after treatment with urea. FRAMPTON and SAUM (1939) and FRAMPTON (1939) reported that dissolving tobacco mosaic virus in urea caused a hundredfold increase in the diffusion constant with no change in infectivity. They interpreted this as indicating that urea disaggregated the virus particles into molecules with a molecular weight of about 100,000 which they suggested were the true virus molecules, the larger particles being simply aggregates of these. However, there was no increase in infectivity such as might be expected from this disaggregation, and later work has not confirmed this view, but shown that urea disrupts the virus. STANLEY and LAUFFER (1939) got no evidence suggesting that the proteins with smaller molecular weights produced by the action of urea possessed any infectivity. They showed that the residual activity in partially inactivated preparations was associated with residual, unchanged virus and not with the small breakdown products. The size of the degradation products varies with the extent of the treatments. MARTIN (1939), from measurements in the ultracentrifuge, gave a molecular weight of 400,000 for the inactivated virus, but by measurements of osmotic pressure STANLEY and LAUFFER

(1939) showed that on continued action of urea it fell to 40,000. They also found that the disintegration depended on the concentration of the urea, the type and concentration of the electrolyte, the pH and the temperature.

BAWDEN and PIRIE (1940) found that the four viruses, tobacco mosaic, potato "X", tomato bushy stunt and tobacco necrosis are all irreversibly denatured by urea. The denaturation is closely linked with loss of infectivity and serological activity. For each virus there is a critical concentration of urea below which there is no irreversible effect on infectivity; this concentration is smallest for potato virus "X" and greatest for tomato bushy stunt virus. In addition to these irreversible effects, however, it is probable that urea can cause changes that can be readily reversed and have no effect on activity. For example, FRAMPTON (1939) found that the viscosity of tobacco mosaic virus solutions was greatly reduced by the addition of 1 M urea. As inactivation occurs only slowly in much more concentrated urea solutions, it is probable that this immediate effect on viscosity is caused by changes in hydration of the virus particles and not by denaturation. The rate of inactivation for all four viruses is greatly increased by the presence of alkali. The rate is minimum at about 20° C and is much increased by cooling to -10° C. The viruses differ in the manner in which they break down as well as in their resistance to urea. The inactivation of potato virus "X" and tobacco mosaic virus is accompanied by the separation of the nucleic acid from the protein and the products of denaturation are soluble in urea solution, whereas the inactivated bushy stunt and tobacco necrosis viruses are insoluble in urea solutions and the precipitates contain nucleic acid.

Other simple organic substances are more efficient inactivators than urea or than the usual solvents such as alcohol and acetone, whose precipitating and inactivating actions are more widely known. Of 15 substances tested ranging from urethane, guanidine and related substances to pyridine, benzoate, salicylate and phenol, all except arginine and nicotine cause inactivation when used in neutral solutions at concentrations of 4 M or less (BAWDEN and PIRIE 1940a). Arginine and nicotine give reversible fibrous precipitates with tobacco mosaic virus; other substances also often act as precipitants without causing inactivation when diluted but when concentrated they dissolve the products of denaturation (BEST 1940). For each substance there is a threshold concentration below which no irreversible changes are produced. As with urea, this threshold is lowest with potato virus "X", intermediate with tobacco mosaic virus and highest with tomato bushy stunt virus. With none of these other substances is there an increase in the rate of denaturation by cooling below 20° C, as there is with urea. In general, the course of denaturation seems similar to that with urea, leading to a separation of nucleic acid from the protein with tobacco mosaic virus and potato virus "X" but not with bushy stunt virus. Sodium dodecyl sulphate, a surface active agent, however, splits off the nucleic acid from all three viruses. Most plant viruses are resistant to the action of strong salt solutions, but STANLEY (1939) states that tobacco ringspot virus is inactivated by ammonium sulphate, which splits off nucleic acid.

Physical treatments which have been found to inactivate tobacco mosaic virus preparations are ultrasonic radiation (high frequency sound waves), and high pressures. TAKAHASHI and CHRISTENSEN (1934) found that clarified sap lost infectivity when exposed to ultrasonics. STANLEY (1934) concluded that the inactivation was not a direct result of irradiation on the virus particles, but was produced by the cavitation of dissolved gas, for he got no inactivation when preparations were irradiated *in vacuo*. Whatever the cause of inactivation, it is accompanied by changes in the virus particles. Liquid crystalline solutions in air immediately lose their birefringence when exposed to ultrasonics and the intensity with which they then show anisotropy of flow decreases with further treatment. The changes in some ways resemble those caused by drying, for the altered, non-infective, part of a preparation does not react with virus antiserum but it precipitates with acid and salts in much the same conditions as active virus. BASSET and others (1938) found that pressures up to 6,000 atmospheres have no effect on infectivity, serological activity or crystallinity, but above 8,000 atmospheres all these properties are lost. The results of denaturation vary with the pressures used. Between 6,000 and 8,000 atmospheres a coagulum of protein free from nucleic acid is produced. As with heating, coagulation follows the course of a first order reaction (LAUFFER and DOW 1941), but denaturation is probably a complex process for loss of infectivity proceeds faster than the formation of a coagulum. At higher pressures, the results of denaturation are soluble, and give sedimentation constants much smaller than that of the intact virus.

THE SIZES OF VIRUS PARTICLES

As yet, little or nothing is known about the sizes of most plant viruses, except that they are too small to be resolved by ordinary microscopic methods. Most have not been studied at all, and of those that have been examined in detail, precise measurements have been made on only very few. Until recently size could only be estimated indirectly, by calculations from measurements made on one or more properties of the viruses, and estimates of the size of the same virus made from measuring different properties have often given widely conflicting results. The next few years will probably bring great advances in knowledge of the exact sizes of virus particles, for with improvements in the techniques of electron microscopy and X-ray analysis direct observation and precise measurements will become possible. These techniques have already been successfully applied to purified preparations of a few viruses. They have been useful in confirming deductions, made from the physical properties of virus preparations, that different viruses have different shapes, that some viruses can occur in particles of varying sizes and in giving the sizes of the particles in such preparations within narrow limits. Such preparations, however, have been subjected to a number of relatively drastic treatments, which may well have affected the sizes of the particles. Until this possibility has been thoroughly tested, it cannot be assumed that measurements on purified preparations give more than an indication of the size of virus particles as they are produced in the infected host. This chapter is a discussion of the methods employed in estimating size rather than an attempt to give final figures for the sizes of the particles.

Filterability:—In most general descriptions of viruses the ability to pass through bacteria-proof filters is usually given a prominent place as a characteristic property. This is understandable because it was the ready filterability of tobacco mosaic virus that enabled IWANOWSKI to distinguish clearly between it and any other known type of pathogen. However, it was in many ways an unfortunate property to be chosen as a criterion of a virus, for in their ability to pass through filter candles different plant viruses show even greater variations than in most of their other properties.

Tobacco mosaic virus, for example, will pass through all grades of Pasteur-Chamberland candles, whereas potato virus "X" will only pass through L_1 , L_3 and L_5 and potato virus "Y" will not pass through L_1 . These differences are obviously not a true reflection of the relative sizes of the different viruses, for an L_1 candle does not retain visible bacteria. They almost certainly result from other factors of which the most important are virus concentration of the infective saps and the electric

charges carried by the virus particles. If the charges are such that they favour absorption on to either the filter or other constituents of the sap then filterability will be greatly reduced. As the filter candles are negatively charged, to facilitate filtration the virus particles should also carry a strong negative charge. All the viruses that have yet been examined have been found to have isoelectric points between pH 3 and pH 5, many of them being insoluble at their isoelectric point. Thus the best conditions for filtration will be obtained by making the preparations as alkaline as possible without inactivating the viruses, for this will increase their solubility and the strength of the negative charge. The expressed saps of many plants have a pH value of between 5 and 6, a condition unfavourable for filtration, unless the virus is like tobacco mosaic with an isoelectric point at about pH 3. The effect of alkali in preventing absorption is clearly shown by potato virus "Y" which in expressed tobacco sap will not normally filter through a thin bed of kieselguhr. But if the sap is adjusted to about pH 8 almost the whole of the virus will pass through. Similarly, although it has already been stated that tobacco mosaic virus will normally pass through the finest grades of filter candles, if the sap is acidified to pH 4 none of the virus will pass through even a thin layer of kieselguhr.

Because of the uncertainty of the pore sizes of porcelain and kieselguhr filters and of their large surfaces which absorb so much virus, in recent years they have largely been replaced by thin filter membranes made from collodion. The advantages of these have been indicated by SMITH (1933), who showed that plant viruses incapable of passing through filter candles with pores ranging from 3 to 6 μ in diameter will pass through collodion membranes with an average pore diameter of 200 $m\mu$.

ELFORD (1933) has described methods whereby membranes with pores of a uniform size can be made by coagulating mixtures of ether, acetone, alcohol and collodion. The constituents must be thoroughly mixed, and then coagulated by carefully controlled heating in draught free conditions. The pore size can be altered within fairly narrow limits by altering the period of heating or by varying the amount of amyl alcohol added to the system. Within wider limits it is altered by adding acetic acid or water. After heating for the requisite time the membrane is flooded with water and then thoroughly washed in distilled water for some weeks before being used. The thickness of the membrane, its water content, and the rate at which water flows through it under a known head of pressure are measured, and the average radius of the pores can then be calculated from the formula

$$= 2l \sqrt{\frac{2qn}{pv}}$$

in which

r = the radius of the pores in cm.

l = the length of the pores (taken as thickness of membrane)

q = volume of water passing in cc./sec.

n = viscosity of water

p = head of pressure in dynes/sq. cm.

v = volume of total pores (taken as water content)

These membranes are now widely used for determining the filterability of viruses, especially for accurate determinations of the filtration end point. Virus suspensions are filtered through a series of membranes with different average pore diameters and the largest which prevents the passage of the virus is taken as the filtration end point. ELFORD states that from this the diameter of the virus particle can be calculated as a fraction of the average pore diameter, the exact relationship between the two varying with the size of the pore as indicated below.

Membrane average pore diameter (A.P.D.)	Diameter of retained particle
10 to 100 $m\mu$	From 0.33 to 0.5 of A.P.D.
100 to 500 $m\mu$	From 0.5 to 0.75 of A.P.D.
500 to 1,000 $m\mu$	From 0.75 to 1 of A.P.D.

Although the method is based on a number of assumptions, such as that Poiseuille's law can be applied to the flow of water through such small pores, and that the capillary tubes run parallel to one another straight from top to bottom of the membranes, it has been used with considerable success with animal viruses which give sharp and reproducible end points. When suspensions are filtered successively through membranes of descending pore sizes, filtrates of almost constant infectivity are obtained until shortly before the limiting pore value is reached. There is then an abrupt fall in the infectivity of the filtrates and membranes with slightly smaller average pore diameters give virus-free filtrates. The filtration end points for different animal viruses vary widely, from 400 $m\mu$ and 250 $m\mu$ for psittacosis and vaccinia viruses respectively to 25 $m\mu$ for foot-and-mouth virus; they suggest that the animal viruses show a steady gradation in size from particles just below the range of microscopical visibility to those only about 10 $m\mu$ in diameter.

The application of filtration to plant viruses has been less successful and the determination of filtration end points has often given conflicting and confusing results. A few viruses, for example, tomato bushy stunt and tobacco necrosis, filter readily to give reasonably sharp and constant end points. Some, for example, tobacco mosaic and potato "X", filter fairly readily, but give widely variable end points, while others, such as potato virus "Y" and tomato spotted wilt, filter only with difficulty through membranes with average pore diameters of from 600 $m\mu$ to 700 $m\mu$ (SMITH and DONCASTER 1936; SMITH and MACCLEMENT 1941).

Some of the difficulty in filtering plant viruses undoubtedly lies in plant sap being an unsuitable medium. The hydrogen ion concentration, especially of sap from solanaceous plants, is high, while the large amounts of protein and mucilaginous substances tend to block the pores and absorb the virus. However, as a few filter well in this medium some properties of the viruses themselves must also determine filterability. It cannot be solely the size of the particle, for, if the filtration end points were a true reflection of size, the particles of potato virus "Y" and tomato spotted wilt virus should be large enough to be visible under the microscope. Isoelectric behaviour is probably an important controlling factor. Tomato bushy stunt and tobacco necrosis viruses are soluble at their isoelectric points and their size

appears to be unaffected by pH changes over the range in which they are stable. The other viruses studied are insoluble at their isoelectric points and their particles aggregate in acid solutions and also readily absorb impurities. The wide differences between the filterability of these different viruses in sap are probably because of differences in isoelectric point and concentration of virus. Tobacco mosaic virus which is isoelectric at pH 3.3 and occurs in high concentrations, filters readily; potato virus "X", which is isoelectric at about pH 4.5 and occurs in lower concentrations, filters less readily; whereas potato virus "Y", which precipitates at pH 5 and occurs at much lower concentrations, filters only with difficulty through membranes with large pores. THORNBERRY (1935) found that filterability was much improved if the leaves were frozen before the sap was extracted, and if pH 8.5 phosphate buffer and nutrient broth were added to the clarified extract. Using this method he found that several strains of tobacco mosaic virus, cucumber virus 1, tobacco ringspot virus and potato virus "X" all gave the same filtration end point of 45 m μ . Similarly, STANLEY (1939) and SMITH and MACCLEMENT (1941) found that tobacco ringspot virus, which is difficult to filter in clarified sap, filters readily and gives a sharp end point at 40 m μ if the sap is adjusted to pH 8.3.

It is apparent that the method of calculating the particle size from the filtration end point can give only approximations and can do this only for particles which are spherical or nearly so. The filtration end point may give some indication of the width of rod-shaped particles, but it obviously tells nothing about the length. Unless the rods are orientated parallel with the length of the filter-pores, however, it is unlikely that filtration will even provide this amount of information, for rigid, elongated particles might block pores many times as wide as the particles. With animal viruses no evidence has been found for the existence of elongated particles, which are so characteristic of many plant viruses. BARNARD has photographed several of the larger animal viruses with the ultra-violet microscope and found that they have approximately spherical particles of a uniform size (Fig. 38). This fact undoubtedly does much to explain the greater consistency of filtration results with animal viruses, and the greater agreement between particle sizes estimated from filterability and other properties, than with many plant viruses.

Of the plant viruses so far studied only those causing bushy stunt, tobacco necrosis and tobacco ringspot give constant filtration end points, and these are the only ones that do not have grossly aniso-dimensional particles. The other viruses give widely different end points in different tests. SMITH and MACCLEMENT (1938, 1941) find four different end points for tobacco mosaic virus, depending on the method of filtration and the preparation. Filtering under pressure, the virus in some samples of clarified sap gives an end point of 50 m μ ; other samples give end points around 180 m μ and after purification by precipitation with acid and salts end points around 400 m μ are obtained. By contrast, if filtration is done in a simple cataphoresis cell, the virus will pass through a membrane with an average pore diameter of only 13 m μ . Similarly, with different samples of potato virus "X", end points varying between 45 m μ and 450 m μ have been found.

BAWDEN and PIRIE (1937*a*) noted that the reduced filterability of the purified tobacco mosaic virus was accompanied by an increase in the amount of anisotropy of flow and by a decrease in the infectivity. To explain these phenomena they suggested that the virus particles in untouched sap were relatively small, but readily aggregated end-to-end to form long rods. A similar explanation was offered to explain the variation in the filterability, optical properties and infectivity of potato virus "X". Later workers (LORING, LAUFFER and STANLEY 1938; LORING 1938*b*) have confirmed this, but find that the viruses purified by high speed centrifugation without the use of strong salt solutions and diluted in alkaline broth, filter under pressure through smaller membranes than virus purified by chemical methods. The variation in filtration

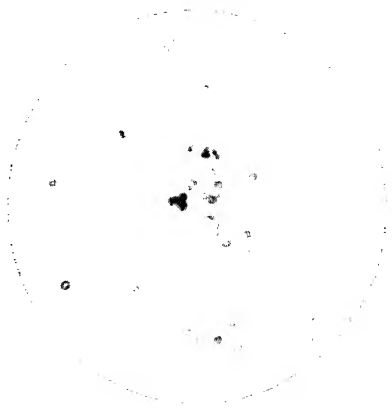


FIG. 38. — Virus of canary pox. Photographed with ultra-violet light, dark ground. $\times 3,200$. (Photograph by J. E. BARNARD).

end points, therefore, probably results from changes in the length of the virus particles. Tobacco mosaic virus is readily orientated when an electric current is passed through solutions. If examined between crossed Nicol prisms, the top-layer fluids immediately light up around the anode when a direct current is applied. This is brought about by the negatively charged particles moving towards the anode and being orientated both by their flow movements and by the increased concentration at the anode. If a filter membrane is interposed between the anode and cathode so that the pores are parallel to the flow movement, then it is to be expected that the orientated rods will pass straight through the pores without becoming jammed. The diameter of the smallest pores through which the particles pass in these conditions is probably approximately equal to the width of the particles. Thus, from the filtration results, it is reasonable to assume that tobacco mosaic particles in neutral solutions have a width of about $13\text{ m}\mu$ but a length which varies and depends partly on the treatments to which the preparation has been subjected.

The three viruses with spherical particles all give approximately the same end point, suggesting that they have diameters of between $13\text{ m}\mu$ and $20\text{ m}\mu$. SMITH and MACCLEMENT (1940) found that sap from plants suffering from tobacco necrosis behaved differently from suspensions of other viruses when filtered through a series of membranes with different average pore diameters. The end point was constant around $40\text{ m}\mu$, but there was always a great loss of infectivity in filtrates through membranes with pore sizes of between $125\text{ m}\mu$ and $250\text{ m}\mu$. This disease can be caused by a number of different viruses and it seems likely that the filtration results are due to working with a culture of mixed viruses which vary from one another in size. The constant end point of $40\text{ m}\mu$ is probably the pore size necessary to

stop the virus with smallest particles, whereas the first drop in infectivity is probably the end point for viruses with larger particles.

Sedimentation, viscosity and diffusion:— In the last few years the high speed analytical centrifuge has been increasingly used in attempts to determine the sizes of virus particles. Apparatus for carrying out such studies has been developed during the last 15 years and has proved of great value in the investigation of proteins and other substances with large molecules. In the machines designed by SVEDBERG high rotational speeds are obtained by driving a large steel rotor by means of a turbine using oil under pressure of several atmospheres. These machines are costly to build and to operate, and recently cheaper centrifuges employing air-driven turbines and capable of equally high rotational speeds have been designed by BEAMS. Both types of sedimentation velocity centrifuge and the sedimentation equilibrium centrifuge have been used in work on plant viruses.

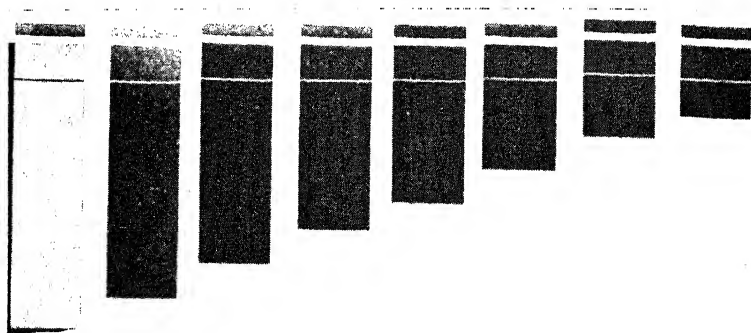


FIG. 39. — A series of sedimentation pictures of tomato bushy stunt virus, 0.27% in 0.02 M acetate buffer. Centrifugal force 20,000 times gravity, 9 minutes between each exposure. Note perfectly sharp boundary in all exposures. (McFARLANE, A. S. and KEKWICK, R. A., 1938, *Biochemical Journal* 32, 1607).

Under ordinary conditions a solute with a greater density than the solvent remains uniformly distributed throughout the solution because the velocity of diffusion of the solute particles is greater than their velocity of sedimentation under the influence of gravity. With suspensions of large or dense particles separation takes place under the influence of gravity unless there is mechanical mixing to assist diffusion in keeping the mixtures homogeneous. The only difference between a solution and a suspension is the stability of the system, which is determined by the weight of the particles. If the gravitational field acting on a solution is increased, *i.e.*, if the solution is centrifuged, particles that would normally have remained suspended indefinitely can be sedimented. By the use of sufficiently high rotational fields it has been found possible to centrifuge out of solution molecules as small as sucrose.

In both types of centrifuge used for measuring sedimentation velocities of molecules in solution, a small volume of solution is en-

closed in a cell provided with plane parallel windows transparent to ultra-violet light, and rotated at high speeds and constant temperatures. If the solvent and solute have different densities there will be a gradual separation of the two because of the intense centrifugal field set up by the rotation. The extent of the separation is measured by photographing through the rotating cell. Two different properties can be utilised for the determination of the concentration distribution of the solute in the cell, namely, the refraction and the light absorption. The second has been used with virus solutions, and is based on changes in the capacity of the fluids to absorb light as the denser particles sediment. The light source chosen is ultra-violet, because this is transmitted by the solvent but absorbed by the viruses. In the sedimentation velocity

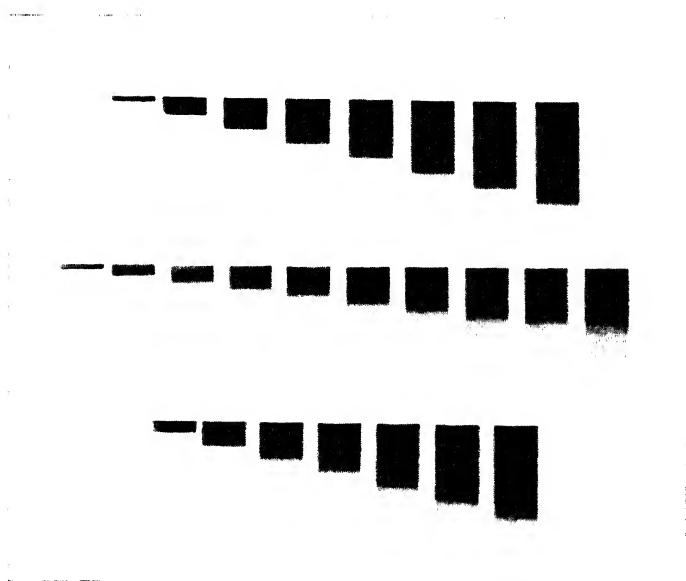


FIG. 40. — Sedimentation pictures of solutions of tobacco mosaic virus. *a.* Virus purified by means of high speed centrifugation and dissolved in water. *b.* After the development of a second component caused by allowing the preparation to stand in the presence of salt. *c.* After more extensive treatment with salt. (STANLEY, W. M., 1935, Harvey Lectures, 1937/38, 170).

centrifuge, centrifugal fields of great intensity are used to shorten the time of observation and to avoid remixing by diffusion. In the sedimentation equilibrium machine, on the other hand, it is not the rate of settling that is measured but the competition between settling and diffusion. The centrifuging is therefore carried on sufficiently long at comparatively low speeds to attain an equilibrium between the two.

If a solution contains molecules of all one size and shape, they will all sediment equally in the centrifugal field. Thus, the fluid at the top of the tube will become transparent to ultra-violet light and the photographs will show a sharp boundary between the lower portion, which still contains solute, and the upper. A series of photographs taken while the cell is being rotated will show this boundary in different

positions, and from the rate at which it is falling the sedimentation velocity of the protein can be measured (Fig. 39). If the fluid contains molecules of different sizes and shapes, these will sediment at different rates and the boundary between the regions absorbing and transmitting ultra-violet light will not be sharp (Fig. 40). If the fluid contains two kinds of molecules, there will be two distinct boundaries. In addition to its value for calculating sedimentation velocities, therefore, the analytical centrifuge also gives valuable information about the homogeneity and the numbers of different sizes of molecules there are present.

The sedimentation velocity reduced to unit centrifugal field, *i.e.*, that causing an acceleration of 1 cm. per sec. and to standard frictional conditions represented by water at 20° C, is called the sedimentation constant. It is expressed as $S_{w,20^\circ} = X \times 10^{-13}$ cm. sec.⁻¹ dynes⁻¹. Sedimentation constants have now been determined for a number of different viruses and virus strains. These are summarised in Table 19.

Table 19:

Virus	Sedimentation constant $S_{w,20^\circ} \times 10^{-13}$ cm. sec. ⁻¹ dynes ⁻¹
Tobacco mosaic	170, 200, 235
Aucuba mosaic	185, 220, 256
Masked tobacco mosaic	211
Cucumber virus 3	173, 200
Cucumber virus 4	173, 200
Potato virus "X"	113, 131
Tobacco ringspot	115
Tomato bushy stunt	146
Tobacco necrosis	112 (Princeton culture) 112 (Potato culture) 112 (Tobacco culture) 49 & 235 (Rothamsted culture)
Alfalfa mosaic	74

The relative sizes of some virus particles as indicated by measurements of sedimentation velocity conflict widely with those indicated by filtration experiments. In theory, both the filtration end point and sedimentation constant are direct functions of particle size, the smaller the particle the smaller its sedimentation constant and the smaller the diameter of the pore necessary to prevent its passage. Yet it will be seen that viruses such as tobacco mosaic, which have given the smallest filtration end points, give the largest sedimentation constants. It will also be seen that the viruses which show anisotropy of flow and which have given a number of different filtration end points, have also been found to give a number of different sedimentation constants. Both methods of measurement, therefore, agree in suggesting that these viruses can exist in particles of different sizes, but it is apparent that neither can be used directly for calculating precise size. The apparent contradiction between the results of the two methods almost certainly arises because different viruses have particles of different shapes and their solutions have widely different physical properties, for variations in such factors affect the interpretation of the results as much as does variation in size.

The translation of sedimentation constants into particle sizes or weights is fairly straight forward if the particles are spherical, although even then some assumptions are needed. For example, it has to be assumed that Stoke's law can be applied to the sedimentation of such small particles as protein molecules and that the specific gravity of the protein in solution is the same as when dry. Nevertheless, the agreement between sizes calculated from sedimentation constants and from other data is sufficiently good with spherical particles to show that the method is reasonably accurate. Only for tomato bushy stunt virus is there positive evidence of spherical molecules. The first measurements of its sedimentation constant were made by MACFARLANE and KEKWICK (1938) who found a value of 146×10^{-13} . This value, however, was almost certainly too high and later workers (LAUFFER and STANLEY 1940; BAWDEN and PIRIE 1942a) have found a value of 132×10^{-13} . If the usual assumptions are made, the diameter of bushy stunt virus particles can be calculated to be $26 \text{ m}\mu$ and their weight equivalent to a molecular weight of 7.4×10^6 . In addition to the sedimentation constant, MCFARLANE and KEKWICK (1938) also determined the sedimentation equilibrium of tomato bushy stunt virus at four different concentrations. At a force corresponding to 150 times gravity at the centre of the fluid columns, equilibrium was almost attained in 48 hours and was complete after 96 hours. From these studies, a mean molecular weight of 7.6×10^6 was calculated. The excellent agreement between this figure and that calculated from the sedimentation constant, assuming spherical particles, strongly supports the view that this virus has spherical particles. All the carefully purified preparations of this virus examined have given sharp boundaries and have showed no phenomena to indicate the presence of particles of more than one size or shape. The boundary remains sharp and the sedimentation constant unchanged when solutions are examined at widely different concentrations at various pH values between 2.5 and 8.5. For particles of such constant size and behaviour, it is perhaps legitimate to use the word molecules. There is a good agreement between the size calculated from sedimentation experiments and from filtration end point determinations. Nevertheless, it would be premature to assume that the precise size of bushy stunt virus is known. In calculating this particle size from the sedimentation constant the effects of diffusion are neglected and the particles are taken as unhydrated. An exact valuation of the weight from sedimentation velocity measurements requires that the diffusion constant be known. NEURATH and COOPER (1940) have found an average value for this in water at 20°C of 1.15×10^{-17} , and if this value is used in conjunction with $S_{w,20^\circ} = 132 \times 10^{-13}$ a molecular weight of 10.6×10^6 is indicated. The discrepancy between this figure and that calculated on the basis of unhydrated particles suggests that the particles may be associated with about 75% of their weight of water.

The estimation of sizes of other viruses which do not show anisotropy of flow is less certain than for bushy stunt virus, for there is no positive evidence about their shape. If they are taken as spherical and unhydrated, and the other usual assumptions are made, figures that indicate the order of the size, even though not the precise size, can be

calculated. Tobacco ringspot virus, with a specific gravity of 1.57, and a sedimentation constant of 115×10^{-13} , corresponds to a particle of diameter $19 \text{ m}\mu$ and weight equivalent to a molecular weight of 3.4×10^6 (STANLEY 1939). Alfalfa mosaic virus, with a specific gravity of 1.48 and sedimentation constant of 74×10^{-13} , corresponds to a particle of diameter $16.5 \text{ m}\mu$ and weight equivalent to a molecular weight of 2.1×10^6 (ROSS 1941). The viruses causing tobacco necrosis, with a specific gravity of 1.35 and sedimentation constants of 112 and 49, correspond to particles of diameters 24 and $16 \text{ m}\mu$, and molecular weights of 5.8×10^6 and 1.8×10^6 , respectively. These figures suggest that the smallest plant viruses yet studied are alfalfa mosaic and the Rothamsted culture of tobacco necrosis, but the value of the measurements on these is uncertain. Alfalfa mosaic virus is unstable and the measurements were made on demonstrably inhomogeneous preparations. Similarly, the measurements on the Rothamsted culture of tobacco necrosis virus were made on preparations that had lost most of their infectivity, although they gave a single sedimentation boundary and were still highly active serologically.

In the first edition of this book it was stated that, in spite of the many confident statements made as to the precise size of particles of tobacco mosaic virus, there was no reason to believe that this was by any means accurately known. This is still very much the position. From the discussion above it will be apparent that even with spherical particles, for whose physical properties there are well established theories, only approximations of size can be made from measurements of physical properties. With non-spherical particles, even the order of size may not be indicated by such measurements. The mathematical difficulties in deriving an equation for the sedimentation of non-spherical particles are so great that it has not yet been done. In attempts to calculate the weights of such particles from sedimentation constants a dissymmetry factor is introduced into the formula used for spherical particles. The first sedimentation measurements were made on tobacco mosaic virus by ERIKSSON-QUENSEL and SVEDBERG (1936), before the rod-like shape of the particles was known. They found a constant of 200×10^{-13} , assumed a dissymmetry constant of 1.3 (a common value for proteins), and calculated a weight equivalent to 17×10^6 hydrogen atoms. Other than as an indication that tobacco mosaic virus particles in purified preparations were larger than any previously recognised protein molecules, however, this figure is of little value, for the dissymmetry factor and the partial specific volume used were both too small. In later work the estimated weights have become larger, but the values given by different workers have varied enormously.

The dissymmetry factor is usually obtained from measurements on sedimentation equilibrium, but, because of the extremely large weight of tobacco mosaic virus particles, this method fails to give satisfactory results. Attempts have therefore been made to calculate it from data on viscosity, diffusion and optical properties. FRAMPTON and NEURATH (1938) and LAUFFER (1938a) interpreted their measurements of viscosity as indicating that the particles were 35-37 times as long as they were thick and calculated a dissymmetry factor of 2.52. Using this in conjunction with $S_{w,20^\circ} = 170 \times 10^{-13}$, LAUFFER deduced a molecular

weight of 42.5×10^6 for cylindrical particles $12.3 \text{ m}\mu$ in diameter and $430 \text{ m}\mu$ in length. LAUFFER further stated that two such particles aggregating end-to-end would give a sedimentation constant of 200×10^{-13} , the average figure found for chemically purified virus. ROBINSON (1939) from measurements of anisotropy of flow, viscosity and deviation of the angle of isocline at various temperatures, concluded that 35:1 was too small for the ratio of length to width and estimated it to be 88:1. He also stated that the equations used in interpreting the results of viscosity measurements could not validly be applied to solutions of tobacco mosaic virus.

NEURATH and SAUM (1938) and FRAMPTON and SAUM (1939) have measured the diffusion constant of tobacco mosaic in attempts to find the particle size. The former give a value of 3×10^{-3} sq. cm. per second and the latter 4.5×10^{-9} . The larger of these constants implies a weight equivalent to a molecular weight of about $1,300 \times 10^6$ and the smaller, of course, implies still larger particles. By applying corrections for a length to width ratio of 37:1, however, the molecular weight can be reduced to 90×10^6 for the larger diffusion constant, and if the diffusion constant is used in conjunction with sedimentation data molecular weights of 68×10^6 and 59×10^6 can be calculated for sedimentation constants of 200 and 174 respectively. It is apparent that with such variations no definite statements can be made about the size of tobacco mosaic virus particles from measurements of physical properties of solutions. The difficulties do not arise solely because the particles depart greatly from the spherical, but because of the fact that there are forces acting between the particles even when they are separated by considerable distances. As a result, solutions do not show normal Brownian movement, they have anomalous diffusion and viscosity and are thixotropic (BAWDEN and PIRIE 1937*a*; ROBINSON 1939; FRAMPTON 1939, 1940). FRAMPTON (1939*a*) has stressed the ambiguity of estimates of size based on data from solutions of tobacco mosaic virus and has especially emphasized the discrepancies between sedimentation and diffusion studies. He considers that any slight agreement between these methods is purely fortuitous, and shows that a molecular weight of infinity can be calculated from the sedimentation constant and one of zero from the diffusion constant. Although this may be an unduly pessimistic conclusion, it is a valuable corrective to the over-confident statements made by some other workers; it also illustrates vividly the difficulties of working with such anomalous material. These difficulties have been enhanced by the confirmation of FRAMPTON's (1939) prediction that solutions of tobacco mosaic virus do not fulfill the requirements essential for the validity of Stokes' law. LAUFFER (1940) has shown that there is interaction between the particles, so that the rate of sedimentation depends greatly on the concentration, the $S_{w,20^\circ} \times 10^{-13}$ increasing by 2.93 when the concentration of the solution is decreased by 0.1 mg. per cc. By extrapolation to infinite dilution, LAUFFER suggests that the true sedimentation constants of the two components usually present in purified preparations are 193×10^{-13} and 216×10^{-13} instead of 174×10^{-13} and 200×10^{-13} given by earlier workers. From these various studies it seems reasonable to conclude that tobacco mosaic particles in purified

preparations are rod-like, can aggregate end-to-end, are at least 40 times as long as thick, and weigh at least as much as 50×10^6 hydrogen atoms, but greater precision is not easily obtained. There is also no positive evidence that these particles are the equivalent of molecules, *i.e.*, that they are the smallest that can exist as active virus.

Other anisotropic viruses have not been studied in any detail, but there are similar difficulties in interpreting measurements on their sedimentation velocities. All these have been found to give more than one boundary after purification, so that it is likely they also aggregate and occur in particles of different sizes. The viruses serologically related to tobacco mosaic virus have been found to give sedimentation constants different from those given by tobacco mosaic virus. It is impossible to suggest at the present time whether these differences indicate real differences in the sizes or shapes of such serologically related strains or whether they are only apparent differences arising because the measurements of the constants have been made under different conditions for different strains. By assuming that particles of potato virus "X" are rod-like, LORING (1938*b*) has calculated a dissymmetry factor and introduced this into the formula for translating sedimentation constants into molecular weights. Using the smallest constant of 113, he estimates a weight equivalent to a molecular weight of 26×10^6 , corresponding to a cylindrical rod of 430 $m\mu$ long and 9.8 $m\mu$ thick. So many assumptions are invoked in reaching this result, however, that there is no reason to believe it even approximates to the real size and shape of the particles.

X-rays and electron microscopy: — The first direct measurements on the width of tobacco mosaic virus, which are still probably the most accurate, were made by X-ray analysis (BAWDEN *et al.* 1936; BERNAL 1938; BERNAL and FANKUCHEN 1937, 1941). Measurements have been made on dried preparations and on solutions of varying concentrations. The pattern obtained falls into two parts: one of large spacings obtained with X-ray cameras working at very low angles, and the other of smaller spacings obtained with high-angle photographs. The first part of the pattern varies, the spacings depending on the *pH* and the concentration of the preparations. At the same *pH*, the spacings increase with increasing dilution, and at a constant concentration they decrease as the *pH* approaches the isoelectric point. Variations in the amount of water separating the virus particles are clearly responsible for these variations. These patterns obtained at low angles reveal previously unsuspected regularities in the structure of solutions, for they show that the distance between the virus particles is inversely proportional to the square root of the concentration by volume, and that the particles are distributed in a hexagonal array so as to fill the available space as uniformly as possible (Fig. 41). This regularity of packing is typical of all orientated preparations of the purified virus, whether as the crystal-like needles produced by precipitation with acid or salts, as solutions or as wet or dry gels. It allows measurements on the effective width of the virus particles. In the dried gel, where it is to be expected that the rods will be packed together as tightly as possible, the particles have an effective width of 15.2 $m\mu$ and effective

area of 201 square $m\mu$. No reflections indicating interparticle distances have been obtained in the direction of the length of the particles, although spacings up to 150 $m\mu$ have been measured. Thus X-rays do not give a figure for the length, but suggest a minimum of about 10 times the width. If it is assumed that the minimum concentration at

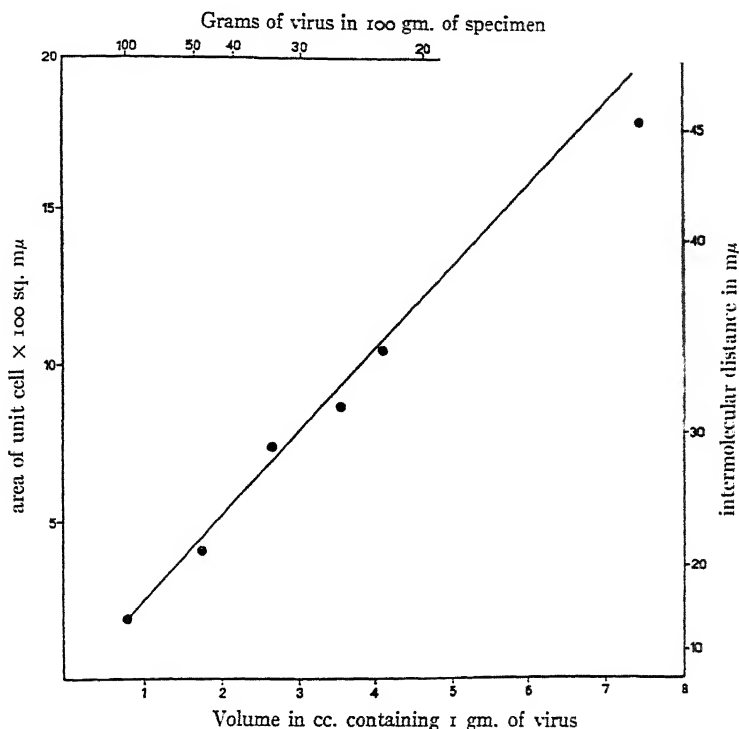


FIG. 41. — Graph showing the relationship between the distance separating virus particles and the concentration of preparations of tobacco mosaic virus (J. D. BERNAL and I. FANKUCHEN, unpublished).

which solutions become spontaneously birefringent is a simple function of particle length, however, an approximation for the length can be made. Taking 1.6% for the critical concentration, and the values for width and area given by X-ray measurements, a length of 1,400 $m\mu$ can be calculated, which suggests that the particles may be about 90 times as long as they are wide (BAWDEN and PIRIE 1937a).

The second part of the X-ray pattern, that of high-angle photographs, results from regularities of arrangement within the particles themselves. It indicates relatively small spacings, which, in contrast to the low angle photographs, are almost independent of the water content. When solutions are dried they change only by about 4%, spacings of 1.15 $m\mu$ decreasing to 1.11 $m\mu$ (BERNAL 1938). From this it seems that the particles in purified preparations of tobacco mosaic virus are rigid and relatively anhydrous. For if, on wetting, they took up the amount of water found in most organisms it is to be expected that there would be a corresponding increase in the internal spacings indicated by X-rays. It is not the fact that tobacco mosaic virus can

be dried and still be infective that distinguishes it from organisms, for some of these withstand drying, but it is the fact that even in solution they occur with little or no internal water that is unusual.

Although the virus particles contain relatively little water they may be associated with water orientated around their surfaces as an "ionic atmosphere" in the manner suggested by McFARLANE (1938) for vaccinia virus. This is suggested by the fact that in neutral solutions the particles are highly charged and by the regular structure of solutions, for such equidistance can only be maintained by definite forces acting between the particles at distances considerably greater than their width. Variations in the thickness of these associated water layers produced by changes in pH might explain the changes in the effective diameters (*i.e.*, the intermolecular spacings) of the particles shown by X-ray patterns of wet gels at different pH values. The presence of such charges might also explain the formation of the fairly rigid gels produced when virus solutions are centrifuged at high speeds, for, in spite of the fact that they all look alike and appear to contain particles of approximately the same length, their water contents can vary between 90% and 70%. And the change in the apparent isoelectric point of tobacco mosaic virus produced by the addition of salts, may result from changes in these charges produced by the presence of other electrolytes. The existence of such layers of charged water around the particles would, however, greatly complicate the theoretical interpretation of the critical concentration at which solutions become liquid crystalline, and the translation of this and of viscosity or diffusion data into particle sizes becomes still more uncertain.

The independence of the two kinds of X-ray pattern has been shown in another way than that one varies with concentration of water while the other remains almost constant. Using orientated pieces of dry gel and photographing with the X-ray beam parallel to the direction of the rods, the scattering at small angles gives a hexagonal pattern of discrete spots. On the other hand, the scattering at large angles gives a pattern consisting of continuous rings. This shows that the rods are arranged with an exact parallelism, whereas there is no such parallelism in the arrangement of the sub-units making up the particles, each of which therefore gives rise to its scattering pattern independently of any other (BERNAL 1938). In other words, the pattern with scattering at small angles with an orientated preparation of tobacco mosaic virus corresponds to the X-ray picture of a single two-dimensional crystal, whereas that at large angles corresponds to the so-called powder photograph given by large numbers of unorientated crystals. The pattern at small angles also reveals regularities only at right angles to the direction of orientation of the particles, whereas that at large angles shows regularities along the length of the particles. The sharpness of these reflections further shows that the particles in purified preparations are sufficiently long for a large number of repeat units to be regularly arranged along each particle.

Surprisingly enough, the clearest photographs of this internal regularity have been given by orientated solutions (*see* Fig. 48). Insufficient work has yet been done for the X-ray patterns to be

interpreted fully, but they are of the same order of complexity as feather keratin. They are a good deal more complicated than the anisotropic muscle-fibre protein myosin, but less so than the fully crystalline proteins such as pepsin (BERNAL 1938). There is a spacing along the length of the particle of approximately $2\text{ m}\mu$ and the virus seems to be composed of piles of sub-molecules of dimensions $2.2\text{ m}\mu \times 2\text{ m}\mu \times 2\text{ m}\mu$. These are rather smaller and less complicated than normal protein molecules, and are themselves divided into nearly identical units with half these dimensions.

So far X-ray patterns have been obtained on preparations of eight different viruses and virus strains. Aucuba mosaic and enation mosaic viruses appear to give intramolecular spacings identical with those of tobacco mosaic virus. The intermolecular spacings are also the same for dry gel, indicating particles of similar widths, but the relative intensities of the lines differ with different strains. No differences have been detected between cucumber viruses 3 and 4 and their intramolecular patterns also resemble those given by tobacco mosaic virus. Their intermolecular spacings in dry gel, however, are smaller, indicating particles with an effective diameter of $1.6\text{ m}\mu$: the lines show even greater intensity differences from those of tobacco mosaic virus than do the lines given by aucuba and enation mosaic viruses. No differences have been found between two strains of potato virus "X". These give a distinct intramolecular pattern from the tobacco mosaic viruses, although it is of the same general type and resembles tobacco mosaic more than any other type of protein that has been examined with X-rays. In virus "X" there is again a strong reflection at $1.1\text{ m}\mu$ perpendicular to the axis, but whereas in tobacco mosaic virus there is one doubled spacing at $2.2\text{ m}\mu$, in this virus there are two reflections, one at $1.65\text{ m}\mu$ and the other at $3.3\text{ m}\mu$. No intermolecular spacings have been found with potato virus "X", so that X-rays give no indication of the size of these particles. The photographs suggest that virus "X" is built up from sub-units substantially the same as in tobacco mosaic virus, but they are arranged differently. With crystals of tomato bushy stunt virus, intermolecular spacings have been obtained. In the wet crystals the particle has an effective diameter of $34\text{ m}\mu$, and after drying one of $27\text{ m}\mu$, a difference that again may result from a surrounding ionised water-layer. This virus also gives an intramolecular pattern showing that the units constituting the spherical particles are also arranged with the fixed regularity of a crystal lattice. This pattern has not been studied in any detail, but is of the same general type as those of potato virus "X" and tobacco mosaic virus, although readily distinguished from them.

No serious attempt has been made to apply ultra-violet light microscopy to the study of plant viruses, but it does not promise to be as successful as with the larger animal viruses, for the plant viruses which have so far been purified all have particles too small to be resolved by the method. In the last few years, however, the recently-developed electron microscope has been increasingly used to photograph plant viruses. The machine can be considered in basic principles as analogous to an ordinary microscope, but the light source and solid glass lenses are replaced by an electron source and magnetic field

lenses respectively. The limitations of the visible and ultra-violet light microscopes are set by the wavelength of light used. The wavelength associated with a beam of electrons accelerated by a potential of 60 kilovolts is only 0.05×10^{-7} mm. whereas for visible and ultra-violet light the wavelengths are about 5000 and 2000×10^{-7} m μ respectively. It is this that gives the electron microscope its greatly increased power of resolution. In theory, with such a wavelength, particles of atomic dimensions should be resolvable. In practice, however, at the present stage of development of the magnetic field lenses, the resolving power falls far short of the theoretical and some of the plant viruses are only imperfectly resolved. To set against the advantages of a greatly increased power of resolution, the electron microscope has several serious disadvantages for studying biological preparations. As electrons are absorbed and scattered by air, a high vacuum has to be maintained throughout the electron path. Excess absorption of electrons by the specimen must also be avoided, for otherwise it will get sufficiently hot for structural changes to occur. The specimens must therefore be prepared as thin films on collodion or some such material and thoroughly dried before they can be examined. It is obvious that such treatment might introduce serious artifacts and that observations made on such specimens do not necessarily give results applicable to the material before treatment.

KAUSCHE, PFANKUCH and RUSKA (1939) first photographed tobacco mosaic virus, and their results fully confirmed the deductions made from X-ray analyses and physical data that the particles were elongated and could occur in particles of different lengths. They found the width to be about 15 m μ and the length either 150 m μ or 300 m μ . Since then measurements have been made by other workers, and, although there is agreement about the width and the fact that particles of different lengths occur, widely different lengths have been found. MELCHERS *et al.* (1940) found that the lengths of particles in their preparation were predominantly 140 and 190 m μ , whereas STANLEY and ANDERSON (1941) found that most of their particles had a length of 280 m μ . The latter workers conclude that this is the length of the normal tobacco mosaic virus particle and from it calculate a molecular weight of 40×10^6 ; they interpret the differences between their measurements and those of MELCHERS *et al.* (1940) on the basis that different strains were being examined and that strains have different particle lengths. FRAMPTON (1942) has challenged these conclusions. He has pointed out that many of the particles in the preparations made by STANLEY and ANDERSON are smaller than their postulated molecules and that some have lengths similar to those quoted by MELCHERS *et al.* FRAMPTON measured the approximate lengths of 159 particles occurring in the photographs published by STANLEY and ANDERSON and plotted a length distribution curve. This clearly showed a bunching of particles at lengths in the regions of 300 , 190 , 150 , 100 and 37 m μ . These lengths are essentially in the ratio of $8:5:4:3:1$, and imply an orderliness that cannot be overlooked. In view of the known tendency of these virus particles to aggregate end-to-end, the simplest interpretation is that the basic particle, or perhaps molecule, of tobacco mosaic virus, is about 15 m μ wide and 37 m μ long, and that the longer particles are



FIG. 42. — Photograph of a dried preparation of cucumber virus 4 taken with the electron microscope. Note the rod-shaped particles of various lengths. $\times 40,000$. (Courtesy of Drs. STANLEY and ANDERSON. Unpublished).

simple multiples of these. This would suggest a molecular weight of only 5×10^6 . However, drying tobacco mosaic virus preparations reduces their infectivity, and it is also possible that the smallest particles are inactive products produced by the disruption of larger particles.

STANLEY and ANDERSON (1941) have also photographed cucumber viruses 3 and 4, tomato bushy stunt virus and the Princeton culture of tobacco necrosis virus. Photographs of the first two clearly show the presence of rod-shaped particles and again illustrate the phenomena of linear aggregation. The particles are all about $15 \text{ m}\mu$ wide but vary in length from $125 \text{ m}\mu$ to over $1000 \text{ m}\mu$ (Fig. 42). The photographs of the other two viruses show no definite rod-shaped particles, but the resolution is not good enough for accurate measurements. They suggest approximately spherical particles of between 20 and $30 \text{ m}\mu$ in diameter.

Various workers have found that the inactivation of virus suspensions subjected to irradiation of different types follows a simple exponential curve, and have concluded that inactivation is caused by absorption of a single ionisation or cluster of ionisations (LEA 1940). This suggests the possibility of using the results of irradiation experiments as a means of estimating the sizes of the virus particles. The estimates are unlikely to be accurate, but the results compare interestingly with those of other methods. With three spherical viruses, those causing tomato bushy stunt, tobacco necrosis and tobacco ring-spot, LEA and SMITH (1942) got results suggesting particles of from one-quarter to two-thirds the sizes indicated by other methods. With tobacco mosaic virus, however, the discrepancy is much greater. GOWEN (1940) calculated that the volume of the particle necessary to cause infection corresponded to a molecular weight of about 5.6×10^6 , and LEA and SMITH's results indicate a value of only half this, *i.e.*, less than one fourteenth the weight of the rod-like particles with lengths of $280 \text{ m}\mu$. This difference again could be explained on the basis that the larger particles are simply linear aggregates of a basic virus particle.

In spite of the large amount of work that has been done on tobacco mosaic virus, there is still no reason to believe that we know the size of the smallest particle necessary to cause infection. All that can be said is that *in vitro* the virus occurs as rigid rod-like particles. The shape of the cross section is unknown, but in neutral solution the width is about $15 \text{ m}\mu$ while the length can vary from an unknown minimum up to over $1000 \text{ m}\mu$. By altering the conditions, for example, by adding acid, the size of the units can be increased and brought up to the range of microscopic visibility. The weights that have been attributed to the particles in neutral solutions have often been called molecular weights, but it is to be doubted if such a variable factor has any claim to this title. A molecule is the smallest particle that can exist as an independent entity, and there is no conclusive evidence that any of the measurements described have been made on homogeneous *molecular* solutions.

The fact that clarified sap gives strong anisotropy of flow shows that many elongated particles are already present, and that these can be made to aggregate linearly to form still longer particles is now generally accepted. It is, however, difficult to gain evidence as to

whether the particles in sap with a sedimentation constant of 170 are actually the smallest that can exist as active virus or whether they are the first most stable type of linear aggregate. BERNAL (1938) concluded that the crystals produced in infected plants are characteristic of smaller and less asymmetrical particles than those found in purified preparations. BALD (1937), from a mathematical interpretation of the dilution curve, showed that the deviations from a linear relationship agree reasonably well with the view that dilution causes a dissociation of aggregates into smaller infective units. Again, in examining concentrated solutions of the virus, no components have been reported with sedimentation constants less than 170×10^{-13} , but in largely diluted solutions WYCKOFF, BISCOE and STANLEY (1937) found considerable amounts of material with relatively small particles. Unfortunately, the material was not tested for infectivity; but it is not unreasonable to assume that it was produced by dissociation of the larger particles. All these observations tend to suggest that many of the particles in infective sap may be molecular aggregates, and, until conclusive evidence against this view can be obtained, it would seem preferable to avoid the use of the word molecule in describing any of the particles of tobacco mosaic virus, or of the other viruses which are known to show the phenomena of aggregation.

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PHYSIOLOGY OF VIRUS-DISEASED PLANTS

Effect on metabolism:—Considering the information that studies on the relative metabolisms of healthy and virus-diseased plants might be expected to give about the methods whereby viruses multiply and produce their characteristic effects, surprisingly little detailed work of this type has been attempted. The results that have been obtained are often conflicting and difficult to evaluate. They are sufficient to show that different viruses affect the physiology of their hosts in different ways, and suggest that the confused nomenclature may be largely responsible for many of the apparent contradictions in published work, different workers having used different viruses or virus strains under the same name.

The two commonest symptoms of virus diseases are chlorosis and a reduction in the size of plants. It is unlikely, however, that the reduction in size results solely, or even mainly, from a reduced rate of photosynthesis. In normal plants the rate is usually limited by light intensity, temperature or carbon dioxide rather than by the chlorophyll content, and the reduced size of virus-diseased plants is rarely proportional to the degree of chlorosis, but varies with different viruses. For example, a strain of cucumber virus 1 causes a general, bright yellow mottle in tobacco but affected plants may be only slightly smaller than normal, whereas strains of tobacco mosaic virus causing little chlorosis may greatly reduce the size. Similarly, the chlorosis of tobacco plants infected with severe etch virus is little greater than that produced by many other viruses, but the growth of such plants is greatly reduced. Here the stunting may be a consequence of the nuclear abnormalities which KASSANIS finds accompanying infection.

Stunting of diseased plants is frequently accompanied by pronounced deformation of the foliage. From tomato plants infected with tobacco mosaic virus KRAYBILL and others (1932) have obtained a non-infectious leaf-deforming substance. This acts as a simple toxin, plants inoculated with it showing symptoms for only a limited length of time, and the extent of the deformity depends on the amount injected. It fails to produce any mottle, and when injected into tomato plants infected with potato virus "X" does not produce streak as does tobacco mosaic virus. The nature of the toxin is unknown. It is not precipitated by protein precipitants, nor is its toxicity destroyed by 2-3 hours' heating at 126° C. It was not found in healthy tomato plants or in plants infected with cucumber virus 1 showing deformities similar to those produced by tobacco mosaic virus. According to THORNTON and KRAYBILL (1934), tomato plants showing severe symptoms produced solely by the injection of the non-infectious toxin resemble plants infected with the virus in that both contain less reducing

sugars, sucrose, starch and hemicellulose but more total nitrogen and nitrate nitrogen than healthy plants.

The manner in which viruses affect chlorophyll is still uncertain, some workers maintaining that the only effect is an inhibition of chloroplast development whereas others consider that mature chloroplasts can be attacked. PETERSON and MCKINNEY (1938) have studied the effect of three strains of tobacco mosaic virus, differentiated by the intensity of mottling produced, and an unrelated virus, probably potato virus "Y", on the plastid pigments and chlorophyllase of tobacco leaves. In contrast to healthy plants, where they found the chlorophyllase content to be directly proportional to the chlorophyll content, the chlorophyllase content of mottled leaves was greatest in those showing the most severe mottles and containing the least chlorophyll. Similarly, the yellow areas from mottled leaves were found to contain less chlorophyll but more chlorophyllase than the green areas from the same leaves. The results they obtained are summarised in Table 20.

Table 20:

Relative chlorophyllase and plastid-pigment content of healthy and mottled tobacco leaves

Enzyme or pigment	Healthy plants	Tobacco mosaic viruses			Potato virus "Y" (?) Mild mosaic
		Common mosaic	Yellow mosaic	Mild dark green mosaic	
Chlorophyllase	100	94.3	186.9	118.5	125.3
Chlorophyll . .	100	74.7	43.3	87.2	72.2
Carotene . . .	100	72.8	39.3	86.7	69.7
Xanthophyll . .	100	74.1	46.9	85.8	75.2

It will be seen that the reduction in chlorophyll content was accompanied by a similar reduction in the content of the yellow pigments, carotene and xanthophyll. PETERSON and MCKINNEY suggest that the chlorosis of diseased plants does not result from an increase in yellow pigmentation but from the partial removal of chlorophyll which in normal plants acts as a masking agent. ELMER (1925), however, also working with tobacco mosaic virus, found approximately a doubling of the carotene content in mottled leaves, although the chlorophyll and xanthophyll were both reduced. These different results cannot be explained, but it is possible that different strains were used or that the plants were grown under different conditions. The increase in chlorophyllase accompanying infection with all except common tobacco mosaic virus, suggests that the viruses have no direct action on chlorophyll, but act indirectly by stimulating the normal plant enzymes.

Additional enzyme activity has been described in plants infected with other viruses. BUNZEL (1913) found that the leaves of sugar beet suffering from curly top have more than twice the oxidase activity of normal leaves. BEST (1937) showed that sap from tomato leaves

infected with tomato spotted wilt virus contains an oxidase catalysing the oxidation of phenol, catechol, quinol and tyrosine in the presence of air. He could not detect this enzyme in the leaves of healthy plants, although it was present in roots. Nor was it found in all species infected with the virus. WOODS (1900) and ROUZINOFF (1930) have also described increased oxidase activity in plants suffering from tobacco mosaic and potato leaf roll. An increase in the diastase content has been reported in sandal suffering from spike disease (SREENIVASAYA and SASTRI 1928) and in tobacco suffering from mosaic (LUDTKE 1930).

Alterations in the carbohydrate content of leaves, and changes in the carbohydrate/nitrogen ratio, produced by virus diseases have been recorded by many workers. These show clearly that some diseases increase whereas others decrease the ratio. For example, the carbohydrate content of tobacco (BAILEY 1924) and tomato leaves (BREWER, KENDRICK and GARDNER 1926) is reduced by infection with tobacco mosaic virus, the reduction being mainly in the polysaccharides. In other virus diseases, on the other hand, especially potato leaf roll (CAMPBELL 1925), there is a large increase in polysaccharides and sugars. DUNLAP (1930) has determined the carbohydrate/nitrogen ratios for a number of plants suffering from different diseases. His results together with those of CAMPBELL for potato leaf roll are summarised in Table 21.

Table 21:
Effect of virus diseases on carbohydrate and nitrogen contents of plants

Host	Disease	Ratios; Diseased plants/Healthy plants		
		Nitrogen	Carbohydrate	C/N Ratios
Tobacco	Mosaic	1.10	0.77	0.71
Tomato	Mosaic	1.09	0.81	0.76
Squash	Mosaic	1.15	0.86	0.76
Pokeweed	Mosaic	1.14	0.78	0.67
Pepper	Mosaic	1.03	0.93	0.88
Cucumber	Mosaic	1.07	0.75	0.70
Peach	Yellows	0.52	1.90	3.62
Aster	Yellows	0.50	1.97	3.79
Plum	Yellows	0.69	1.36	1.99
Ragweed	Yellows	0.45	1.71	2.24
Potato	Leaf roll	0.78	2.31	3.00

The nitrogen and carbohydrate were both measured as a fraction of the dry weight.

DUNLAP suggested that virus diseases could be divided into two classes, one accompanied by a decrease in nitrogen and increase in carbohydrate, and the other by an increase in nitrogen and a decrease in carbohydrate. Although this may be true, there is as yet insufficient evidence to prove it. It is possible that DUNLAP himself was working with fewer viruses than he suspected, for the mosaics of tomato, tobacco, pokeweed and pepper may all have been caused by strains of tobacco mosaic virus. Other work, however, tends to support the suggestion. ROSA (1927) found a decrease in nitrogen in tomato plants

infected with sugar beet curly top virus to be accompanied by an increase in carbohydrate. Similarly, in spinach suffering from blight, TRUE and HAWKINS (1918) describe an accumulation of carbohydrates and this disease has also been found to reduce the nitrogen content (JODIDI and others 1920).

That many diseases with mosaic symptoms are accompanied by a reduced carbohydrate content is also fairly well established. Their effects on nitrogen are less definite. The increases found by DUNLAP are small relative to the changes in carbohydrate, and CORDINGLEY and others (1934) have recorded similar increases of approximately 10% in the total nitrogen content of tobacco plants suffering from mosaic. STANLEY (1937) has claimed larger increases in tobacco plants infected with some strains of tobacco mosaic virus. He found that two strains of cucumber virus 1, potato virus "X", severe etch and tobacco ringspot viruses all caused a decrease in the total protein, but he gives no data on total nitrogen. By contrast, he stated that tobacco mosaic and aucuba mosaic viruses stimulate protein production, so that, although infected plants are smaller than normal, their total protein and nitrogen contents are higher than those of healthy plants of the same age, sometimes being twice as great. MARTIN, BALLS and MCKINNEY (1938), however, have failed to confirm this, and point out that STANLEY's results merely show an increase in the amount of extractable protein in infected plants and not in the total protein or nitrogen. These workers have measured the total protein and total nitrogen of three tobacco varieties when healthy and when infected separately with three strains of tobacco mosaic virus and have been unable to detect any significant changes. As the clarified sap of infected plants can contain from two to five times the amount of protein in clarified healthy sap, depending on the methods used for clarification, this suggests that the additional soluble protein is produced at the expense of healthy plant protein which is normally insoluble.

The accumulation of carbohydrates that occurs in the leaves of many virus-diseased plants cannot be taken as evidence of increased photosynthesis. Indeed, BARTON-WRIGHT and M'BAIN (1932) state that in potato plants suffering from leaf roll, where the accumulation of starch in the leaves is probably greatest, photosynthesis is much reduced. The effect is more likely to result from some hindrance to the translocation of carbohydrates. Similar accumulations accompany some deficiency diseases, and can be produced in the potato by damage to stem-bases or by removing tubers as they are formed.

QUANER (1913) first showed that the phloem in potatoes suffering from leaf roll becomes necrotic, and suggested that this was the cause of starch accumulating in the leaves. MURPHY (1923), however, pointed out that rolling of the leaves and starch accumulation begins soon after infection, long before any phloem necrosis becomes obvious. He and other workers have suggested that phloem necrosis is a secondary effect, the carbohydrates remaining fixed in the leaves in an insoluble form because of some changes in enzymatic activity. THUNG (1928) obtained no evidence to support this view. He found that the starch in rolled leaves readily changed into sugars when the leaves were

placed in the dark. Also, in comparing the carbohydrate contents of rolled and healthy leaves, he found that the former usually contain more sugar as well as more starch. THUNG considers that some function of the phloem is affected and reduces translocation. He recognised the fact that translocation is inhibited before a visible change in the phloem can be detected, and suggested that the necrosis and leaf-rolling are both symptoms of some other change in the phloem. BARTON-WRIGHT and M'BAIN state that the sugar of translocation in the healthy potato is sucrose whereas in leaf-roll plants it is hexose, and suggest that translocation in the diseased plants is restricted to diffusion through the ground parenchyma. They consider that the reduced photosynthesis results from starch accumulation, and that



FIG. 43. — Leaf of Arran Victory potato suffering from foliar necrosis, photographed (a) on a panchromatic plate and (b) on an infra-red plate. Green parts of the leaf reflect infra-red rays whereas the necroses absorb them. (BAWDEN, F. C., 1933, *Nature* 132, 168).

the main reactions in rolled leaves are conversion of starch to hexose, hexose to sucrose and sucrose back to starch. Although the carbohydrate metabolism of potato plants is so altered by leaf roll, BARTON-WRIGHT and M'BAIN (1933*b*) found only slight effects on the protein. Nitrate tended to accumulate in the petioles and the protein content was reduced in leaf-roll plants, but they concluded that the formation of protein and other nitrogenous compounds proceeds in essentially the same manner in diseased as in healthy plants, although at a slower rate. SCHWEIZER (1930), on the other hand, contends that phloem necrosis and accumulation of starch result from a derangement of protein metabolism. That tubers from leaf roll plants often remain hard and undecomposed in the soil when used for seed has frequently

been noted. A statement of SCHWEIZER may explain this. He states that infected tubers germinate later than healthy ones, and that the proteins pass into the young shoots much sooner than the carbohydrates. This complete migration of protein into the shoots stops diastatic activity in the tubers, so that starch remains unaffected and the flow of sugars to the developing shoots is arrested.

The fact that other viruses which have no demonstrable effect on the phloem also inhibit the translocation of carbohydrates from the leaf suggests that in leaf roll there may be additional factors. Many viruses causing mosaic symptoms have been found to give starch-iodine local lesions (*see* page 27), and the production of these depends on starch being retained longer in virus-containing than in virus-free areas. As the starch is removed from the virus-free areas rapidly, the effect can hardly be attributed to a disturbance of the phloem, but presumably results from an alteration in the infected parenchymatous leaf cells which either slows down the transformation of starch into sugars or prevents the diffusion of sugars. BOLAS and BEWLEY (1930) find that tomato leaves after inoculation with aucuba mosaic virus at first actually lose starch more rapidly than healthy leaves when placed in the dark, except at infection points where starch is retained. The effect is not restricted to local lesions. As tobacco mosaic virus becomes systemic in tobacco plants, the first areas to be invaded can be clearly distinguished by placing the plants in the dark until the starch has left the virus-free areas, and then staining with iodine. The effect of these viruses, therefore, is clearly on the parenchymatous cells and not on the conducting tissues. Late in infection, however, conditions are different from those in potatoes with leaf roll, for instead of starch accumulating in the mottled leaves there is a reduction of about 50% in the carbohydrate content (DUNLAP 1931). BARTON-WRIGHT and M'BAIN (1933a) have also found that translocation of carbohydrates is reduced in Arran Victory potato plants suffering from paracrinkle, although the phloem of these plants shows no necrosis. Late in infection the hexose, sucrose and starch contents of crinkled leaves are higher than those of healthy, but the increases are considerably less than in leaf roll plants. No differences were detected between the carbohydrate contents of virus-free President plants and those carrying the paracrinkle virus.

In Arran Victory plants suffering from crinkle caused by a simultaneous infection with potato viruses "A" and "X", BARTON-WRIGHT (1941) describes a different effect. The carbohydrate metabolism is but little affected, whereas the total nitrogen and the protein nitrogen in the diseased plants are considerably higher at all times than in the healthy plants. An increase in both nitrogen and carbohydrate contents is described by SREENIVASAYA and SATRI (1928) in sandal suffering from spike disease. Deficiency of calcium is also stated to be a characteristic feature of this disease, and it is suggested that this may be responsible for the reduced translocation of sugars. SREENIVASAYA (1930) finds mannitol in all diseased plants but never in healthy ones, and suggests that this sugar may be a specific product of virus-activity.

THUNG has examined the quantities of CO₂ given off by detached

leaves from healthy and leaf-roll potatoes when floated on water in the dark. He found the respiration of the diseased leaves was higher per gram fresh weight as well as per gram dry weight, and suggests that the greater concentration of respirable sugars is responsible for this rather than an increase in enzyme activity. WHITEHEAD (1931) has examined the relative respiration rates of healthy and infected potatoes at various stages in the life-cycle and confirmed the higher



FIG. 44. — Photographs of the under surface of a tobacco leaf showing systemic invasion by the virus causing tomato spotted wilt; *a*, showing the necrosis when photographed by daylight on a panchromatic plate; *b*, showing fluorescence when photographed under filtered ultra-violet light, with an aesculine filter placed between the leaf and camera to absorb any reflected ultra-violet. (BEST, R. J., 1936, Austral. J. Exp. Biol. & Med. Sci. 14, 199).

rates for the latter. He found that infected immature tubers respired more than healthy ones at first, although towards the end of dormancy they respired slightly less. In the sprout stage they also respired less, but immediately leaves were produced their respiration rate was higher than that of healthy sprouts and continued so throughout the growing period. In the young plants, therefore, respiration was greater than in healthy plants before the leaves showed any signs of rolling. Nevertheless, WHITEHEAD (1934) concludes that the increased rate

of respiration is not directly related to the presence of the virus but to the available amount of respirable substrate, such substances accumulating in the leaves of infected plants at a very early stage of development. He found that the accumulation could be delayed by continuous exposure to light of low intensity, when the rate of respiration of diseased plants approximated to that of healthy ones. The anaerobic respiration of infected and healthy tubers was the same.

Increased respiration rates are not restricted to plants with virus-diseases in which there is an accumulation of carbohydrates. DUNLAP (1930) found that young leaves infected with tobacco mosaic virus respired at a greater rate than healthy ones, although mature diseased leaves respired more slowly. CALDWELL (1934), on the other hand, found that the CO_2 output of tomato leaves infected with aucuba mosaic virus was consistently higher than that of healthy leaves. This was so whether the respiration was measured in terms of initial dry weight, residual dry weight or of residual nitrogen, and whether CO_2 output was measured in oxygen or in nitrogen. As the carbohydrate content of these diseased leaves is less than that of the controls, CALDWELL attributes the greater respiration to an increased activity of the plant's enzyme system.

The chemical changes which occur in plants showing necrotic symptoms are still incompletely understood. They are frequently accompanied by the disappearance of carbohydrates from the neighbourhood of necrotic spots. This is particularly obvious in potato tubers taken from plants suffering from top-necrosis, in which the necrotic phloem elements become surrounded by cork cambium and the adjacent cells lose their starch grains. In the leaves, necroses of two kinds occur, one showing externally as white spots or rings and the other as black or dark brown spots. In necrotic areas of the first type, all the cell contents have disappeared leaving merely dead, empty cells. In areas of the second type, some of the cells may also be empty, but others contain large masses of darkly staining, gum-like materials, which are rich in pectin and possibly tannin. The cell walls are suberised or cutinised and considerably thickened, the reactions resembling those caused by wounding. The two kinds of necrosis behave differently when photographed with light of different wavelengths. The white necrotic areas, such as those caused by virulent strains of potato virus "X" in tobacco, behave like the normal green leaf tissue and do not absorb infra-red rays. The black necrotic areas, on the other hand, such as those found in top-necrosis or foliar necrosis of the potato, absorb infra-red rays strongly (Fig. 43) (BAWDEN 1933). BEST (1936) has found that the dark necrotic areas produced by tomato spotted wilt virus in tobacco, and by tobacco mosaic virus in *N. glutinosa*, contain a substance which fluoresces strongly in ultra-violet light (Fig. 44). BEST states that the fluorescence results from the production in large quantities of a water soluble, organic substance in the infected plants reacting with necroses. This substance is stated to be present in small quantities in healthy plants. Whether or not fluorescence follows infection depends on the type of lesion produced, for the production of this substance, like that which absorbs infra-red radi-

ation, is not a specific property of any particular virus. For instance, leaves from tomato plants suffering from spotted wilt do not fluoresce, nor do mottled leaves from plants infected with tobacco mosaic virus. Similarly, if leaves from tobacco plants infected with tobacco ringspot virus are examined in ultra-violet light, those showing necroses fluoresce whereas those showing less severe symptoms do not.

Movement of viruses in infected plants:—In most plant virus diseases infection soon becomes systemic. There are exceptions, but it is usual for viruses to spread from a single entry point throughout the vegetative parts of plants, the first symptoms often appearing on young leaves at the apex far from the site of inoculation. The first effects of other pathogens are usually restricted to areas around the infection points. Viruses may owe this characteristic feature to the small size of their particles which allows them to diffuse relatively rapidly and to form stable suspensions or solutions. The exact mechanism of virus movement and the path taken are still controversial questions.

It is now generally accepted that viruses move only through living tissues. The inhibition of movement by dead leaf tissue is clearly indicated by the localisation of virus in leaves which react to infection by producing necroses. The necrotic local lesions produced by tobacco mosaic virus in *N. glutinosa*, for example, contain appreciable quantities of virus but this does not move into the intervening green areas however long the leaves remain on the plant. Continuity of living tissue in the stem is also

necessary for movement of virus from one part of a plant to another. By killing off portions of tomato stems with steam, CALDWELL (1930) was able to restrict infection with aucuba mosaic virus to the inoculated part of the plant, the virus being unable to cross the piece of dead stem. If one leaf above the steamed portion was inoculated all the others above became infected, whereas those below remained healthy, and *vice versa*. The plants remained quite turgid showing that movement of water was unaffected, so CALDWELL concluded that the virus does not normally enter the xylem of infected plants. Later (1931) he claimed that if the virus was experimentally introduced into the xylem of tomato plants it remained there, the plants showing no symptoms unless the vessels were mechanically damaged. GRAINGER (1933) and MATSUMOTO and SOMAZAWA (1933), on the other hand, conclude that tobacco mosaic virus enters all tissues including the xylem, but their evidence is inconclusive. CALDWELL



FIG. 45.—Leaf of tobacco plant inoculated with diluted sap from plants infected with yellow tobacco mosaic virus. Treated with iodine eight days after inoculation. The uninfected leaf tissue has stained darkly, and the local lesion and the path taken by the virus down the vein to the petioles show clearly. (SAMUEL, G., 1931, Ann. Appl. Biol. 18, 494).

(1931, 1934a) concluded that movement of virus was largely independent of phloem as well as xylem, and that it takes place readily through any living tissue. He considers that it is independent of the translocation of elaborated food materials, the virus moving at a more or less uniform rate in all directions and increasing in amount as it passes from cell to cell. GRAINGER also concludes that spread is independent of mechanical carriage in the transpiration or translocation streams, although from a consideration of his data which show a greatly accelerated rate of movement on the fourth day after infection, it is difficult to see why he should.

Other workers on the movement of tobacco mosaic virus suggest two different kinds of movement: one a slow diffusion from cell to cell in the inoculated leaves, and a second more rapid movement in the phloem once the virus has reached this tissue. HOLMES (1930) found that the virus at first increases at the inoculation site and moves only slowly. Then suddenly after a few days the virus can be detected throughout the stem, roots and upper leaves. Using the starch-iodine technique (1931, 1932), he showed that systemic infection first occurs on and around the veins of young leaves. Cutting the interveinal areas of leaves had little effect on the rate of movement, but cutting through main veins slowed it down appreciably. The distribution of virus in partly shaded plants suggested that the movement was correlated with the translocation of elaborated food materials. Also using the iodine technique, SAMUEL (1931) obtained similar results with a strain of tobacco mosaic virus causing a yellow mottle in tobacco. He found that the virus spread slowly from the inoculation point until it reached a vein, when it rapidly passed into the petiole and appeared again in the veins of young leaves (Figs. 45, 46).

SAMUEL (1934) has examined the movement of tobacco mosaic virus in tomato plants and found further evidence suggesting that virus first moves slowly from infection points through the leaf tissues and then rapidly along the conducting tissues. In this work plants were inoculated on one terminal leaflet, then cut up into portions at regular intervals and the portions tested for the presence of virus. Before being tested, the stem portions were incubated for some days to ensure that any virus they contained would multiply sufficiently to cause infection. No virus passes out of the inoculated leaflets during the first three or four days, but this period of slow movement is followed by one of rapid movement through the petiole and stem. After entering the petiole the virus first passes down the plant to the roots, where it can be detected within 12 hours of entering the petiole, and a day later it can be detected in the young leaves at the apex. This rapid movement is not dependent on continuous multiplication of virus as suggested by CALDWELL and GRAINGER, for portions of the plant far away from the inoculated leaflet often contain virus while portions nearer do not. In other words, virus particles must pass through pieces of stem but leave them virus-free. In the earliest stages at which virus enters the stem, virus particles may be separated by distances of several centimetres. SAMUEL frequently found pieces of stem 2.5 cm. long to be virus-free, although pieces above and below contained virus. SAMUEL suggests

that this rapid movement takes place in the phloem and is correlated with the transport of elaborated food materials from the leaves. He found that developing fruit trusses above the inoculated leaf became infected at the same time as the roots, whereas leaves adjacent to the

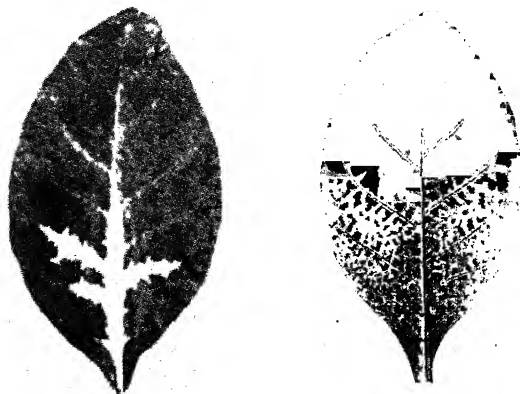


FIG. 46. — Two leaves from the centres of plants becoming systemically invaded with yellow tobacco mosaic virus. Before staining with iodine these showed clearing of the veins. The darkly staining parts at the tips are not infected, the virus being restricted to the areas around the veins which are differentiated by the iodine staining. (SAMUEL, G., 1931, *Ann. Appl. Biol.* 18, 494).

trusses remained virus-free for days or weeks. In pot plants, after the initial infection of the developing leaves at the apex and of the roots, the older leaves become successively invaded. All the leaves of small, vigorously growing plants are soon infected, but it takes three weeks for a medium sized plant to be invaded completely and as much as two months for plants in the fruiting stage. Fig. 47 is a diagrammatic representation of the invasion of a medium-sized tomato plant by tobacco mosaic virus. The mature leaves of large plants growing in the field remained free from virus up to periods of three months after infection, except for a limited infection along the main veins.

CAPOOR (1939) has confirmed SAMUEL's results with tobacco mosaic virus and obtained similar results with a number of others transmitted by rubbing. After infecting a single leaflet, there is a waiting period with all before virus can be detected in the petiole, but after this the roots and apex soon become infected. The length of time taken by the different viruses to move out of the inoculated leaflet varied from 2.5 days with potato virus "Y" to 6 days with aucuba mosaic virus. KUNKEL (1939) found that tobacco mosaic virus on entering the stem usually moved upwards and downwards equally, though occasionally movement was only in one direction. Sometimes the virus moved more than 7 inches in an hour. KUNKEL, like SAMUEL and CAPOOR, found that in the early stages of entering the stem, virus particles were often separated by considerable distances, *i.e.*, long pieces of virus-free stem were often found interspersed irregularly be-

tween portions containing virus. It seems, therefore, that virus particles can pass through long chains of cells without infecting or multiplying in them, indicating that movement of individual particles over long distances does occur and that multiplication and distribution is not an autocatalytic chain process.

BENNETT (1939, 1940) has shown that the direction and rate of movement of tobacco mosaic virus can be altered at will by changing the conditions that affect the translocation of food. He finds that movement towards the apex is accelerated by darkness or defoliation, whereas movement towards the base is slowed down. In *Nicotiana glauca* plants with tobacco scions grafted at the base and apex, virus moved from the top to the bottom graft in 6 days, whereas in 7 out of 10 plants virus introduced into the bottom graft did not infect the upper in 200 days. When the tops of these plants were defoliated, however, movement of virus upwards from the bottom graft was rapid. When the phloem continuity in tobacco plants was broken, tobacco mosaic virus passed through, but its movement was retarded. In *N. glauca*, on the other hand, breaking the phloem prevented the passage of tobacco mosaic virus, but not cucumber virus 1. In earlier work on leaf curl and mosaic of raspberry, BENNETT (1927) had suggested that movement of the viruses depended on the movement of food in the phloem. He showed that these viruses produced symptoms on the new growth of canes inoculated and passed into the roots within a few days or weeks, but, under normal conditions, non-inoculated canes on the same plant remained healthy. In the following spring the viruses entered the non-inoculated canes soon after growth began. If non-inoculated canes were pruned sufficiently to stimulate the growth of lateral buds, however, they became infected in the current season. Ringing the stems to remove phloem prevented the passage of both viruses.

The experiments of BENNETT (1934, 1937) with sugar beet curly top virus provide the most precise information on the conditions determining and the tissues involved in virus-movement. This virus has been shown to occur in the phloem in much greater concentration than in other tissues, and not to move across parts of tobacco stems from which the phloem has been removed. Movement towards the roots in plants growing normally is faster than upward movement. When *N. glauca* plants were grafted at the top and bottom with tobacco scions, virus introduced into the basal scion did not reach the upper in 300 days. On the other hand, virus introduced into the upper scion caused infection in the lower in 30 days. If plants inoculated in the basal scion were defoliated, however, infection of the upper scion occurred in 18 days. Similarly, in sugar beet which were split into three shoots in such a manner that they remained united by the lower end of the tap root, virus introduced into one shoot did not move into the other portions in periods of over 100 days. But if one of the non-inoculated shoots was defoliated, or placed in the dark, it became infected in a few days. This virus passed out of green leaves inoculated at the distal end in as short a time as 4 hours, but it took 21 days to move from etiolated leaves. When infected etiolated leaves were again illuminated, however, the virus moved into the petioles within a day or

two. From these results it seems quite clear that in both beet and tobacco plants the entrance of the curly top virus into shoots can be inhibited by supplying conditions favouring the rapid synthesis of carbohydrates and facilitated by making conditions unsuitable for

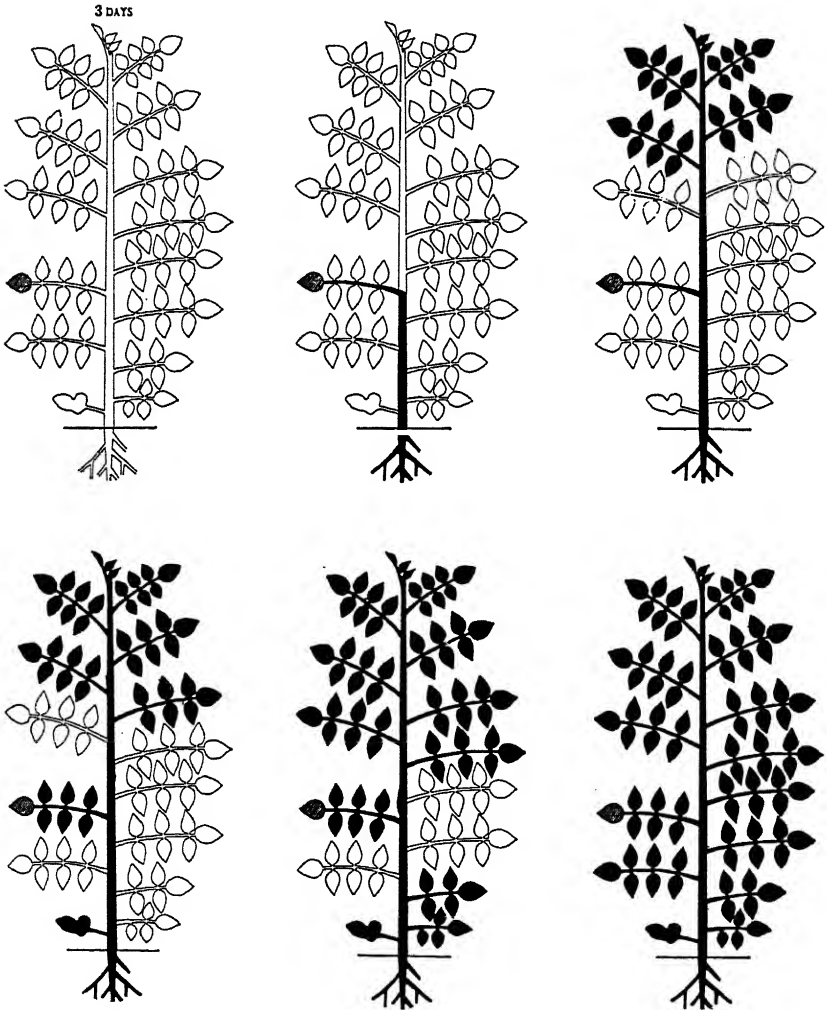


FIG. 47. — Diagram showing the progress of tobacco mosaic virus (in black) through a medium-sized, young tomato plant. Based on tests made with Dwarf Champion plants about 15 in. high, growing in 6 in. pots in an unheated greenhouse. The inoculated leaflet is shown shaded. (SAMUEL, G., 1934, *Ann. Appl. Biol.* 21, 90).

photosynthesis. Thus it is highly improbable that the virus moves steadily through the plant along a virus-concentration gradient, but more likely that it is carried mechanically in the stream of elaborated food materials. In all the virus diseases yet examined virus has been

found to move fairly soon from inoculated leaves into the roots. By contrast, PRICE (1938) found that if the roots of tobacco plants were inoculated with tobacco mosaic virus the virus did not pass out and infect the tops in periods up to three weeks, although it multiplied readily in the roots. FULTON (1941) found that the direct inoculation of roots with several different viruses produced infection, but the virus remained restricted to the roots. Upward movement in the roots was exceedingly slow, whereas downward movement was as rapid as in stems. BENNETT (1940) found that cutting off the tops of plants greatly increased the rate of movement of tobacco mosaic virus from roots to the tops. Similarly at Rothamsted, we find that the aerial portions of tobacco and tomato plants are more likely to become infected with tobacco mosaic virus as a result of root infection if the leaves are removed or if the plants are kept in the dark for some days. In good growing conditions with strong light, we have kept plants for months with a high virus content in the roots but with their tops virus-free. All the phenomena clearly indicate that viruses can move only with difficulty against the main stream of elaborated food materials whereas they move rapidly in the same direction.

The constant association of phloem necrosis with sugar beet curly top and some other virus diseases is often brought forward as evidence for the presence of virus in the phloem and for this tissue being involved in the translocation of viruses. This argument, however, is of doubtful value. In many potato varieties infected with virus "Y" necroses originate in the cortex, the whole of which may be affected, while the phloem remains normal. Thus, if the suggestion be adopted that the tissues in which necroses originate are those in which the virus moves, it becomes necessary to assume that virus "Y" differs from all those previously studied and moves through stems in the cortex.

Curly top virus moves from its entry point into leaves much more rapidly than tobacco mosaic virus or others transmitted by rubbing. SEVERIN (1924) detected curly top virus 18 cm. from the insect's feeding place within half an hour, and BENNETT (1934) found the rate of movement downwards through sugar beet leaves to be 2.5 cm. per minute. In tobacco, where translocation of carbohydrates is less than in sugar beet, the virus moved more slowly, approximately 1.5 cm. per hour. STOREY (1928) found that maize streak virus, which is also transmitted by leafhoppers, travelled 40 cm. from the infection point in 2 hours. These rates contrast remarkably with the periods of days required for mechanically-transmitted viruses to pass out of inoculated leaflets. They approximate much more closely to those indicated by SAMUEL for tobacco mosaic virus on the fourth day of infection in tomato plants, and are of the same order as those found by plant physiologists for the rate of movement of metabolites through the phloem.

It is generally accepted that infection with curly top and maize streak viruses occurs only when they are placed directly into the phloem. With tobacco mosaic virus, on the other hand, infection occurs if the virus is merely introduced into epidermal cells. It seems reasonable to assume that the wide differences between their initial

rates of movement is a result of the viruses being placed in different tissues. The viruses transmitted by leafhoppers will immediately be placed in the tissues in which there is a constant flow out of the leaf, whereas tobacco mosaic virus must pass through layers of parenchymatous cells before entering the vascular tissue. How this cell to cell movement takes place is unknown, but it is most likely by diffusion along a concentration gradient set up by virus-multiplication. UPPAL (1934) has measured the length of time taken for tobacco mosaic virus to pass from the upper epidermis to the lower epidermis of leaves and calculates a rate of 8μ per hour, a value not incompatible with movement by diffusion. The fact that different viruses take appreciably different times to pass out of inoculated leaves into petioles, suggests that they either multiply at different rates or have different sizes and hence diffuse at different rates.

As viruses do not pass through semi-permeable membranes some explanation is needed to explain their ready diffusion from cell to cell. The obvious explanation, and the one most frequently made, is that the viruses move from cell to cell along the connecting protoplasmic strands, the so-called plasmodesmata. It is, of course, difficult to get direct evidence for this, but it would explain some experimental results. Many insects become infective by feeding on plants for too short a time for their stylets to have reached the phloem. As their stylets most often take an intercellular course to the phloem this seems to indicate that the viruses can be acquired from the intercellular spaces. DRAKE, MARTIN and TATE (1934) state that the contents of cells adjacent to the stylet tracks produced by aphids feeding in onions are often partially extracted without the cell walls being punctured. This suggests that the cell contents and presumably the infecting virus have been withdrawn along the protoplasmic connections. The apparent stomatal-infections that have been claimed by some workers could also be explained by virus coming into contact with the plasmodesmata and so gaining an entry to uninjured cells.

Although it cannot be claimed that the problem of virus-movement in plants has been solved, the evidence available strongly suggests that movement of two kinds occurs. The first is a slow movement found in parenchymatous tissues. This is probably partly determined by virus concentration, and consists of a diffusion of virus particles through infected cells and from cell to cell, probably along the plasmodesmata. The second is a rapid movement, by which the viruses are distributed from one part of the plant to another. This appears to be independent of the virus concentration, and to take place in the phloem, the rate and direction being determined greatly by the translocation of elaborated food materials.

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THE CLASSIFICATION OF VIRUSES

Naming and grouping. — The most urgent necessity for some accepted scheme of classification of viruses undoubtedly lies in the chaotic state of virus nomenclature, for, although classification and nomenclature are not identical, they are so closely interrelated that it seems clear that only on the basis of their relationship with one another can some order be brought into the naming of viruses. In the early work, when a virus was found infecting a host not previously known to be susceptible to any virus, or one was found causing a set of symptoms distinct from any previously recognised disease, it was usually described as a new virus. It was then given a name derived from the host plant in which it was found and the main type of symptom produced, without any serious attempt to relate it to any other known virus. At first this was reasonable, for it was generally believed that viruses produced constant symptoms and had limited host ranges. Even after it was known that many viruses have wide host ranges, and can exist in strains of varying virulence and cause very different symptoms in different hosts, names were still applied on the dual basis of host and symptoms. As a result confusion has arisen, because different workers have often used different names for the same virus or the same name for different viruses. The name potato mosaic, for example, has been applied to so many different viruses that it has become meaningless except to describe a condition of the host. JOHNSON (1925) was the first to realise that clinical conditions were quite unsuitable for identifying viruses, and he pointed out that tests of properties *in vitro* were often better and more reliable. JOHNSON suggested giving viruses numbers instead of symptom names, but he retained the name of the host plant in which the virus was first found or in which it was considered to be most important.

The continued use of the name of the host plant for the most prominent part of the names of viruses has almost inevitably led to a grouping of viruses on the basis of their association with a particular plant. It has culminated in a scheme of classification proposed by SMITH (1937), in which viruses are divided into 51 groups. Each group takes the generic name of a host plant, for example, there is a *Cucumis* group, a *Nicotiana* group, a *Solanum* group, and so on, the individual members of each group being distinguished by taking different numbers. Thus the name received by any virus depends merely on the host in which it is found and on how many others have previously been found in this host. There are already 18 viruses in the *Solanum* group, so that the next discovered affecting the potato will automatically become *Solanum virus 19*, regardless of whether its properties more nearly resemble those of *Solanum virus 1* (or, for that matter, *Cucumis virus 1*) than those of *Solanum virus 18*. Except for a few viruses which may

have limited host ranges, it is obvious that the position occupied by any virus is purely fortuitous, depending only on which host it happened to be first encountered. The viruses causing aster yellows, tomato spotted wilt, and cucumber mosaic, to quote only a few with wide host ranges, might almost equally as well be placed in any other of the groups as in the *Callistephus*, *Lycopersicum* and *Cucumis* groups, where they now respectively find their homes. It is not surprising, therefore, that the groups resulting from this scheme contain viruses with remarkably different properties. To take only one example: the *Cucumis* group contains two viruses which differ in almost every way except that they are both transmitted mechanically. One has a thermal inactivation point of 55° C, is inactivated in a few days *in vitro*, is transmitted by aphids and has an enormous host range; the other has a thermal inactivation point of 90° C, remains active *in vitro* for years and has not been transmitted by aphids or to any plant outside the *Cucurbitaceae*.

A grouping that gives such different viruses the same generic name is clearly not a classification of viruses; it is merely a system of nomenclature of viruses, following the classification of plants. The artificiality and dangers of such methods of grouping can perhaps best be illustrated by applying a similar one to fungi. *Ophiobolus graminis* would be placed, together with *Erysiphe graminis* and *Puccinia graminis* in the *Triticum* group, and *Phytophthora infestans*, *Rhizoctonia solani* and *Synchytrium endobioticum* would all come together in the *Solanum* group. By contrast, two fungi as closely related as *Puccinia Prunispinosae* and *Puccinia graminis* would be as widely separated as the *Rosaceae* and the *Gramineae* in HUTCHINSON's classification of plants. Grouping on the basis of host plant may provide a convenient method of indexing or cataloguing viruses, but sufficient has probably been said to show that it can never supply anything except a very misleading system of classification.

At first sight a scheme proposed by HOLMES (1939) looks much more like a true classification than does SMITH's, for the host plants have no place in the grouping and naming. The 89 viruses treated are separated in 10 families and named on a Latin binomial-trinomial system that suggests relationship of the same type as genus, species and variety in the classification of plants and animals. On closer examination, however, it becomes apparent that this scheme is little better as a classification than SMITH's, for many of the viruses placed together in one family have few or no properties in common. Nothing like sufficient use has been made of the existing knowledge concerning the properties of the viruses, and symptomatology is the main basis of separation, with the result that viruses often have the same generic name when the evidence available clearly shows them to be unrelated. For example, 53 of the viruses are placed in the family *Marmoraceae*, which contains a single genus *Marmor*. Such widely different viruses as tobacco mosaic, potato "X" and "Y", tomato bushy stunt and tobacco necrosis are all found with the same generic name of *Marmor*. This is definitely misleading by suggesting fairly close relationships between viruses known to differ in almost all their properties. The five quoted differ in the shape of their particles, their stability *in vitro*, the

manner in which they are inactivated, and no one is serologically related to any other. Yet their names and their grouping in HOLMES' scheme indicates that they are as closely related to one another as is tobacco mosaic virus to cucumber virus 3, although these two are known to share antigens, to be equally stable *in vitro* and to have particles of similar shapes and sizes.

The separation into families on the basis of symptomatology can only give a classification of diseases and not one of viruses, for these cause different symptoms in different hosts and different strains of one virus are largely recognised because they cause distinct symptoms in the same host. As a result, there is little doubt that many viruses would be equally at home in one family as another. The sole difference between the viruses placed in the *Marmoraceae* and the *Annulaceae* is that plants infected with the latter eventually recover and show a type of non-sterile immunity. Now this is partly a property of the host plant and environment, and is also a feature of infection with viruses other than the tobacco ringspot viruses, which are the only ones placed in the *Annulaceae*. Even with these viruses, recovery is incomplete and the non-sterile immunity does not extend to all hosts; cowpea, for example, either gives only necrotic local lesions or else dies with a systemic necrosis when infected with tobacco ringspot virus. Tobacco plants infected with some virulent strains of potato virus "X" recover from the necrotic stage of infection in much the same way as when infected with tobacco ringspot virus, whereas plants infected with less virulent strains continue to show definite mosaic symptoms. On such a basis of classification, therefore, some strains of potato virus "X" should be placed in *Annulaceae* and others in *Marmoraceae*. Actually, there is no evidence that the viruses placed by HOLMES in *Annulaceae* differ fundamentally from many of those in *Marmoraceae* any more than these differ from one another, and it is clear that the criteria for grouping do not represent natural relationships between the viruses themselves. The grouping of value that has been achieved is that of strains under one specific name, but the value of this is minimised by the use of the same generic names for viruses with widely different properties.

The great failing of HOLMES' scheme is the paucity of genera within families and the setting up of some families on insufficient and artificial grounds. VALLEAU (1940) has pointed out that the scheme gives an erroneous impression of known relationships and that the genus *Marmor* contains several unrelated viruses while there is little or no reason for the existence of *Annulaceae* as a separate family. He proposes the abolition of HOLMES' trinomial system of naming strains, and classifies the commonly occurring viruses of the tobacco into 7 genera, *viz.*: *Musivum* (tobacco mosaic type viruses), *Murialba* (cucumber virus 1 and potato virus "Y"), *Annulus* (ringspot viruses), *Foliopelis* (etch viruses), *Tractus* (streak viruses), *Lethum* (spotted wilt virus) and *Ruga* (leaf curl virus). This scheme has the advantage over any other that the names suggest no false relationships, for only viruses with closely similar properties are given the same generic name. Its disadvantages are that only a few viruses have been considered and some of the genera contain only one species, for such strains as tobacco mosaic virus and aucuba mosaic virus are given the same name, *Musi-*

vum tabaci. Thus the scheme, in spite of the improvement over earlier ones, is still largely one of convenient nomenclature rather than of classification.

Identification of virus strains:— During the last 10 years, two methods of identifying viruses as related strains have been increasingly used and are now almost the accepted criteria of close relationships. These are based on two kinds of immunological relationship: firstly, the ability of viruses to react with each other's antisera, and secondly, their ability to protect plants against one another. The serological method has much to recommend it, for it is independent of the host plant and is based on structural similarities in the virus particles themselves. It also has the virtue that it can give extremely rapid identification. Detailed serological tests necessitate skilled handling and the careful preparation of stable antigens, but CHESTER (1937) has shown that viruses can be identified with certainty, by unskilled workers, as strains of a type virus merely by determining the ability of antiserum to the type virus to flocculate crude infective sap.

Serological tests, however, have their limitations and dangers. As yet they have failed to give results with many viruses on which they have been tried. This cannot be taken as evidence that such viruses differ in any fundamental way from those that give good precipitin reactions. Indeed, it is known that some viruses, which fail to give such reactions in crude or clarified infective sap, are actually strains serologically related to others which give these reactions. Sap from tobacco plants infected with mild etch virus, for example, gives no reaction with antiserum prepared against severe etch virus or against its homologous antiserum. Sap from tobacco plants infected with severe etch virus, on the other hand, precipitates well with antiserum prepared by injecting rabbits with sap containing either severe or mild etch viruses. Thus these two viruses are serologically related, but the relationship is revealed by the precipitin test only when mild etch virus is used as an immunising antigen and severe etch virus as a test antigen. A positive precipitin reaction can only be obtained with an infective sap if the virus concentration exceeds a minimum value. In tests with purified viruses, this minimum varies from about .02 mg. to .005 mg. per cc. with different viruses, depending partly on the shape of the virus particles. The fact that the precipitin test has failed to give results with sap from plants infected with some viruses is almost certainly because the virus content is below this necessary minimum. With sap from plants infected with an avirulent strain of *Hyoscyamus* virus 3, and with some tobacco necrosis viruses, this fact is easily demonstrated. The saps are not flocculated by specific antisera, but if the viruses are concentrated several times by precipitation and resolution in a smaller volume of water, a positive precipitation test is then obtained. When other viruses, which occur in sap at high dilutions, have also been concentrated, their serological reactions and relationships will also probably become capable of testing. Such work will be too laborious to be of practical value in the routine diagnosis of naturally infected plants, but the results will be of great value for purposes of classification. Where the precipitin test can be directly

applied, it provides the quickest and most reliable method of indentification, and already more than ten serologically distinct virus groups have been separated (Table 22).

Table 22:

Viruses grouped according to their serological reactions

The viruses in each group are precipitated by each other's antisera, but not by antisera prepared against viruses in other groups.

1. *Tobacco mosaic group*:
Common tobacco mosaic and masked tobacco mosaic viruses.
JENSEN's isolates causing yellow mottles and necroses in tobacco.
Tomato aucuba and enation mosaic viruses.
Japanese petunia mosaic virus.
Cucumber viruses 3 and 4.
2. *Potato virus "X" group*:
SALAMAN's H, G, L, S, and N strains of virus "X".
Potato viruses "B" and "D".
American potato mottle and ringspot viruses.
Hyoscyamus virus 4.
British Queen streak virus.
3. *Potato virus "Y" group*:
Potato virus "Y".
American potato veinbanding virus.
Stipple streak virus.
Hyoscyamus virus 2.
Potato virus "C".
4. *Cucumber virus 1 group*:
Cucumber virus 1, including PRICE's isolates Nos. 2, 6 and 8.
VALLEAU's delphinium virus.
5. *Hyoscyamus virus 3 group*:
WATSON's virulent and attenuated strains of Hyoscyamus virus 3.
6. *Tobacco etch group*:
Tobacco etch and severe etch viruses.
BLAKESLEE's Z-mosaic virus of *Datura*.
7. *Pea mosaic group*:
OSBORN's pea viruses 2 and 3.
8. *Tobacco ringspot group*:
WINGARD's tobacco ringspot virus.
Tobacco yellow and green ringspot viruses.
Tobacco ringspot virus 2.
9. *Tomato bushy stunt virus*.
10. *Tobacco necrosis group A*:
Potato culture.
Princeton culture.
Tobacco VI culture.
11. *Tobacco necrosis group B*:
Tobacco cultures I and II.
12. *Tobacco necrosis group C*:
Rothamsted culture.

Because of their instability, or because they occur in a form unable to be precipitated by antiserum, some viruses may remain incapable of testing with the precipitin reaction. It is possible that the application of more sensitive serological reactions, such as complement fixation, may throw further light on the relationships of these. Positive tests can be obtained by this method with one-tenth the amount of virus needed for the formation of a visible precipitate, and the method

also works well with antigens which are not rendered insoluble by combination with antibodies. Although there is little doubt that serological techniques can still be much more widely employed in testing virus-relationships, the methods can lead to false interpretations unless carefully applied. A negative result with a heterologous antiserum, for example, is of no significance unless a positive result is obtained with the homologous serum in the same conditions, for it may merely imply that the fluids undergoing test contain insufficient virus to give a positive reaction. A too small virus content, rather than lack of serological relationship, most probably explains the failure of sap from plants with lily mosaic and celery mosaic viruses to precipitate with cucumber virus 1 antiserum, for these viruses are almost certainly related strains. On the other hand, when negative results are obtained in reciprocal or mirror tests, *i.e.*, when the two viruses do not react with each other's antisera but do with their own, the negative result is more reliable as evidence of no serological relationship than a positive result is of relationship. For the reliability of positive results depends on the certainty that the antigens used for both immunisation and testing are pure cultures. If the antigen used for immunisation is a mixture of two viruses, then sap from plants infected with either will react with the antiserum and suggest false relationships. Some workers have claimed serological relationships between cucumber virus 1 and potato virus "Y", and between potato viruses "Y" and "A", but others have been unable to confirm them, and it is possible that the use of antigens containing mixtures of viruses is responsible for these claims. If the viruses used for immunisation and testing are carefully selected, however, there should be no real danger from such complications. Where possible it is best that they should be propagated from single local lesions, or passed through some plant known to be immune from any suspected contaminant, or transmitted in some specific way. The advantages of the first method have been shown with viruses causing tobacco necrosis. Plants suffering from this disease are often infected with serologically distinct viruses, all of which will react with antisera prepared against the bulk culture. If single lesion cultures are made, however, the viruses propagated from these often fail to react with each other's antisera. An indication that mixed antigens or antisera are being used in serological tests is often supplied by the formation of double zones of optimal precipitation, for with preparations of single viruses only one zone is usually formed.

The plant protection technique gives less rapid results than the serological method, but it is at the moment more generally applicable and has already been widely used for the identification of differently named viruses as related strains. The principles underlying the technique are described in Chapter 6. It has the advantage over serological methods that it is independent of the virus content of expressed sap, and it can be applied to viruses whose other properties have not been investigated because of difficulties of transmission. For example, by intergrafting between peach trees suffering from yellows, little peach and rosette, three diseases caused by viruses not transmitted mechanically, KUNKEL (1936) showed that the first and second are caused by strains of one virus whereas the third is caused by an

unrelated virus. The method gives most definite results when it is applied to two virus strains only one of which produces local lesions in the host. If the host is first inoculated with the strain that gives only systemic symptoms and then inoculations are made at intervals with the second, the growing protective effect as the first multiplies is clearly shown by the progressive fall in the numbers of local lesions. Provided that the virus strains being tested produce sufficiently distinct symptoms, however, the method can be reliably used when only systemic symptoms are produced, and when transmission is made by insects or grafting instead of inoculation. Protection is often less complete when grafting is used, but provided adequate healthy control plants are used there is rarely any difficulty in demonstrating that there is protection. This is especially so as two unrelated viruses often give a more severe disease, or one of a different type, from that given by either virus alone, whereas with virus strains the resulting conditions will be less severe than that produced by the more virulent strain alone, even if protection is not complete. If two strains differ widely in their rates of multiplication and eventual concentration in systemically infected plants, or if the susceptibility of the host plants alters with age, there may be incomplete protection, but the results should not be misleading if adequately controlled with healthy plants. One disadvantage of the plant protection method is that it cannot be applied to viruses which have no common host or which produce only local lesions. The relationships between cucumber virus 3 and tobacco mosaic virus, for example, or between some viruses causing tobacco necrosis, are readily shown by serological methods but could not be demonstrated by plant protection tests.

In general, where serological and plant protection tests have been applied to the same viruses they have given the same results. The few disagreements probably result from the use of mixed antigens or from the fact that sap from plants infected with some strains contained too little virus to cause precipitation. An unusual result has been described with the serologically unrelated viruses potato "Y" and severe etch. Plants infected with potato virus "Y" are susceptible to infection with severe etch virus, but the second seems to be able to suppress the first, and as might be expected from this, plants suffering from severe etch are unaffected by inoculation with potato virus "Y".

Further bases of classification:—The immunological properties are by far the most valuable for the identification of unknown viruses as strains of a type virus. By combining the results of serological and plant protection tests, the number of immunologically distinct virus groups already separated could be considerably increased over those shown in Table 22. Although this would still fall short of a complete systematic classification, it would represent an advance of considerable importance, for some form of nomenclature could be fitted to this grouping that would do much to reduce the present confusion. It seems likely that some form of binomial nomenclature will prove most attractive to virus workers, for this is probably the simplest way of expressing relationships and its use with plants and animals has worked reasonably well. Each group could take some distinctive generic name,

and each virus placed in the group could take a specific name from the most characteristic property that distinguishes it from others in that group. Up to now most strains have been differentiated because they cause distinctive symptoms, so that these specific names would mainly be derived from symptomatology. Where other properties have been used, however, as with potato yellow dwarf (BLACK 1941) and tobacco necrosis (BAWDEN and PRIE 1942) viruses, the specific names should indicate these.

All the viruses that can be grouped together by either of the immunological tests have many similar or identical properties. From the work already done, however, it can be seen that the viruses falling into one group do show varying degrees of inter-relationships. Some of the strains of tobacco mosaic virus, for example, are so alike that their ability to infect a certain host or cause a certain type of symptoms is the only available criterion for their identification. Detail cross absorption experiments show that such strains are not antigenically identical and that the viruses identified as distinct strains for clinical reasons are different proteins, although they share most of their antigens. The differences between some other strains, however, is considerably greater; these have only few antigens in common and they can be differentiated fairly readily by straight-forward serological tests. With strains sharing only a few antigens it is also usually possible to find significant differences between such characters as isoelectric point, filterability, etc. The specificity of the immunological tests, however, is so exact that their value is restricted to the grouping of strains around a type virus. While this in itself is a great advance, larger groupings are essential, and other bases for division and arranging into families are needed, before any true classification can be arrived at.

Although work of various kinds clearly shows that viruses show all the gradations of inter-relationships indicated in the classification of organisms by the terms varieties, species, genera, families and orders, our knowledge of many viruses is still too fragmentary for any final conclusions on the best bases for division and grouping. It is, however, clearly desirable that any lasting system of classification should be based on intrinsic properties of the viruses themselves, and not on those of host plants. Differences in pathogenicity, as with bacteria and fungi, are of great value in differentiating between related strains of the same virus, but similarities of host range and symptoms are entirely inadequate to serve as bases for primary sub-division. No other pathogens have been, or could be, classified systematically on clinical grounds, and there is no reason to think that viruses will be more amenable to this method. Even with the bacteria, in which observable structural differences are slight, morphology supplies the basis for primary sub-divisions. The structural differences of viruses, of course, cannot be observed directly, but with the extension of X-ray analysis to the measurement of larger spacings and the improvements in the electron microscope, detailed work on the structure of viruses becomes increasingly possible. As all the viruses so far isolated are nucleoproteins, the methods that have been of value in classifying proteins are also likely to be of value in making major groups of viruses. Already those

methods have shown clearly that different viruses differ in shape, that they precipitate in different conditions and forms, and that they vary in the manner in which they are inactivated by certain treatments. Some of these properties can only be tested on highly purified preparations, but for others infective sap might be used. For example, with viruses that occur too dilute for examination directly in polarised light, the precipitation test gives an indication of the shape of the virus particle, for rod-like viruses give a distinctly different kind of precipitate from spherical ones. Then inactivation by heat is clearly of two kinds: with one type of virus it is closely linked with denaturation and has a large thermal coefficient, whereas with others it occurs without denaturation and has a small thermal coefficient. For viruses which are not transmitted mechanically, such methods now appear quite unsuitable, but it is likely that in the future techniques will be found enabling their properties to be tested in detail. Until this is done, such viruses can conveniently be put in a group together, with a name chosen to show that this is the reason for putting them there and not that their properties are sufficiently known to be certain that they are fairly closely related.

It has often been suggested (*e.g.*, ELZE 1931; STOREY 1931) that methods of transmission, and especially insect vectors, might furnish methods for classification, but the limitations and dangers of such methods are now fairly obvious. We know little about the reasons for the failure of mechanical transmission with viruses, and it is likely that they differ for different viruses. As some viruses are transmitted by many different insects, and the aphid *Myzus persicae* is already known to transmit more than twenty viruses, a grouping based on the vector would be almost as misleading as one based on host plants. Depending on the length of time for which the vectors remain infective, viruses can conveniently be divided into two, but it is doubtful if this division could be applied as a major basis for classification (WATSON and ROBERTS 1940). Little is known of the properties of persistent viruses, but the few which have been studied differ widely. The non-persistent viruses do appear to form a more uniform group, but even with these, groupings on other bases than relationships with vectors seem preferable. This is shown by recent work at Rothamsted on potato viruses "C" and "Y". These two viruses are obviously related strains, for they have antigenic groups in common, protect plants against one another, and have many identical properties *in vitro*. Yet, in conditions in which 100% infection is regularly obtained with potato virus "Y", we have been unable to transmit potato virus "C" by *Myzus persicae* or *Aphis rhamni*.

Already, by examining the known properties of viruses, a few groups approximating to families can be seen emerging. The many strains of tobacco mosaic virus, tomato aucuba and streak viruses and cucumber viruses 3 and 4 form one. Potato viruses "C" and "Y", the strains of cucumber virus 1, *Hyoscyamus* virus 3, tobacco etch virus and sugar beet mosaic virus form another, and the viruses causing bushy stunt and tobacco necrosis a third. These groupings can be derived from many fundamental properties of the viruses themselves, and as our knowledge of such properties are extended to other viruses,

the number of natural groups will automatically increase. Although it will be a long time before the properties of many viruses will be determined, this is no valid objection to their use as a basis of classification. A classification of fungi on systematic lines was attempted long before all the members could be placed in one or other of the main groups. Those about which insufficient was known were placed in a group apart and clearly labelled *Fungi imperfecti*. There seems no good reason why viruses should not be treated similarly.

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THE CONTROL OF VIRUS DISEASES

Therapeutic treatments:—Two features of virus diseases that separate them sharply from most other infectious diseases, namely, the systemic infection and the persistence of viruses in the hosts, also complicate their control. The initial infection usually passes unnoticed, for often no striking lesion is produced at the site of infection, and the first symptoms are not seen until after the virus has spread through the plant. Thus protection of a whole plant by removing the portion first to become infected is impracticable. The virus also usually persists in the infected plants for as long as any vegetative parts remain alive. The symptoms may fluctuate widely; under some growing conditions they may disappear completely, and in some diseases there is always a first acute reaction, which is followed by a phase in which the plants may be almost indistinguishable from uninfected ones. In spite of these apparent recoveries, it is extremely rare in nature for plants to recover in the sense that they cease to be infected. Plants which have once shown symptoms of a virus disease, whether or not they continue to do so, therefore remain as sources of infection, and in general the only sound precautionary measure is to destroy them.

For a few virus diseases, however, therapeutic measures have now been devised, which destroy the viruses without killing the hosts, and result in complete cures. The possibility of such treatments was indicated by work on two suspected virus diseases of the sugar cane, the sereh disease (HOUTMAN 1925) and the chlorotic-streak disease (WILBRINK 1923; MARTIN 1930; BELL 1933). Sugar cane cuttings can survive immersion in water at 52° C for periods up to an hour, and it was found that cuttings from diseased parents usually grew into healthy plants if held in water at this temperature for 20–30 minutes. The first disease known definitely to be caused by a virus for which a cure was claimed was peach yellows. KUNKEL (1935) reported that trees could be cured by growing them for a fortnight or more at temperatures around 35° C. Later (1936*a* and *b*) he found that peach trees suffering from little peach, red suture and rosette were also cured by this treatment, although trees suffering from mosaic were not. The period of treatment necessary was longer for large than for small trees and the virus in the tops was destroyed before that in the roots. There seems no doubt that the trees are cured and the viruses are actually destroyed by these treatments. Scions from the treated trees produce no symptoms in healthy plants when grafted to them but when the treated trees are grafted with infected scions they develop typical yellows once more. Thus it seems improbable that the cure is merely because of masking of symptoms or because of the heat treatment causing the virus to change to an attenuated form, as it does

with tobacco mosaic virus. KUNKEL also found that peach trees could be cured by hot-water treatment similar to that used for the sugar cane. Whole trees in the dormant state were cured by immersing them for about 10 minutes in water at 50°, while bud sticks were cured at lower temperatures; at 34° C they were cured in 5 days, at 42° C in 40 minutes and at 50° C in 4 minutes. The viability of the buds was unaffected by any of the exposures needed to destroy the viruses.

Heat treatment for peach trees has also been successfully used by HUTCHINS and LA RUE (1939) and by HILDEBRAND (1941) for curing phony disease and yellow-red disease. KUNKEL (1941) has extended the work to other diseases and hosts, and by growing plants at 40° C for two weeks has freed periwinkles, *Vinca rosea* and *Nicotiana rustica* of aster yellows virus. Periwinkles were also cured by immersion in water at 45° C for a few hours. Heat treatment is unlikely to be generally applicable for curing plants of virus diseases. Various workers have found that when virus-infected potato tubers and bean seeds have survived heat treatments, the viruses have also survived. It is obvious that the higher the temperature that a plant will survive the greater are the chances of a cure by heat, and that the success of the method depends on the difference between the resistances to heat of the viruses and the hosts. For viruses with high thermal inactivation points, short exposures to heat are unlikely to have any effect because the hosts are likely to be more susceptible than the viruses. For those viruses which have large temperature coefficients of thermal inactivation, long exposures to lower temperatures are also unlikely to have value, as inactivation proceeds only slowly except at temperatures near the thermal inactivation point. With viruses such as bushy stunt, however, which have high thermal inactivation points but small temperature coefficients of thermal inactivation, long exposure of infected plants at moderate temperatures might achieve success.

The possibility of curing by chemotherapy has been raised by work of STODDARD (1942), also on the peach. He states that buds from peach trees suffering from X-disease can be cured simply by soaking them in water solutions of quinhydrone, urea and sodium thiosulphate. The success of such treatments again will depend on the relative resistances of the host proteins and the viruses to the substances used, and with increasing knowledge of the substances that at low concentrations denature viruses *in vitro*, further curative measures of this sort can be anticipated. No one has yet reported the curing of infected plants by irradiation, although this would also seem worth investigating, for *in vitro* many viruses have been found to be fairly rapidly inactivated by various kinds of radiation.

The discovery of effective therapeutic treatments will have obvious practical applications. They will make possible the regeneration of many varieties of vegetatively-propagated plants, such as potatoes and strawberries, which are now universally virus-infected, and will be invaluable for the building up of nuclear stocks of healthy propagating material. The treatment of scions before use would also prevent the spread of viruses that now frequently occurs because of the unwitting use of infected material. However effective such treatments may be, they are unlikely to be practicable on a large scale or against all dis-

eases, and the main principle of controlling virus diseases must lie in prevention rather than cure.

General control measures:— Spread of virus diseases from infection foci within a crop is much more rapid than spread from plants outside. The simplest precautionary measure that can be advocated, therefore, is the removal of all infected plants from a crop immediately they become obvious. This will not stop spread within the crop completely, for plants can act as sources of infection before they have been infected sufficiently long for symptoms to be visible, but it will greatly reduce it. Also, it is of no value for keeping carrier varieties free from virus-infection as these show no symptoms when infected. Thus, other measures to prevent the entry of viruses into the crop should also be employed. The chief of these is the isolation of the crop from any other likely to be infected, for the further that infection has to travel the less likely is the crop to become seriously affected. Different varieties of the same plant should not be grown together, unless they are known to react clearly to the same viruses, for apparently healthy individuals of one may be infected with a virus that causes a serious disease in another. All weeds should also be kept down as these may act as hosts for the viruses, often without themselves showing any very definite symptoms. All groundkeepers (individuals left over from previous crops) should also be removed and destroyed, for these are one of the commonest sources of early infection within a crop. Isolation and frequent inspection, together with rogueing of diseased plants, are often impracticable on crops grown on a large scale for consumption, but they should always be adopted where crops are being grown primarily for the supply of propagating material. Even with plants raised from true seed, in which seed transmission is rare or unknown, it is a wise precaution. Sufficient examples of transmission through the seed have been described to show that this may be of some importance, for the occasional plants that become infected in this way are sources of infection for the rest of the crop immediately they are above ground. With crops that are propagated vegetatively, or with those such as the bean in which transmission through the seed is a regular occurrence, the necessity for maintaining a high degree of health in parent stocks is obvious.

The ideal method for the control of virus diseases would be to raise varieties of crop plants immune from them, but unfortunately there is little evidence with most of our crops that parents with the requisite genes exist. Nevertheless, the production, in America, where all commercial stocks of potatoes are infected with potato virus "X", of a new seedling (U. S. D. A. 41956) immune from this virus, shows that this method has potentialities even with what appeared to be most unpromising material. In the absence of any true immunity, there are still ample opportunities for the plant breeder to produce new varieties valuable in combatting losses from virus diseases. The different varieties of many species of plants react in widely different ways to infection with the same virus; one variety may be killed outright, a second suffer a chronic, crippling disease, while a third may show only slight symptoms and a fourth act as a carrier. The use of carrier-

varieties is already widely practiced with some crops. For example, in some parts of England the Huxley variety of strawberry has almost entirely replaced Royal Sovereign, because it carries yellow-edge virus, which cripples Royal Sovereign. Commercial varieties of hops and potatoes, which act as carriers of one or more commonly occurring viruses, are also widely grown.

The most striking success of plant breeding for resistance to virus diseases has been in the control of sugar cane mosaic. The production of the resistant P. O. J. series of varieties has rendered this disease a minor problem in Java and elsewhere, where previously it was so serious as to threaten the existence of the crop. Similarly, the great losses of sugar beet in the U. S. A. caused by curly top have been largely prevented by the raising of resistant lines of beet (U. S. Nos. 1, 33, 34). The superiority of these over the old intolerant varieties has been repeatedly demonstrated in field tests where they have often yielded more than five times as much. From the published descriptions of resistant varieties it is often difficult to assess the precise significance to be attached to the term resistance. It is usually ascribed to varieties that do not suffer appreciably in the field, but whether this is due to a true resistance to infection or to ability to tolerate infection is often not clear. Carrier-varieties undoubtedly are of great value in reducing losses, but whenever possible resistance to infection is to be preferred. Carrier-varieties, especially of vegetatively propagated plants, rapidly become infected, and if they are at all widely grown they soon supply enormous reservoirs of infection. This is of little importance if the host range of the particular virus is limited to one crop and if all the varieties of that crop tolerate infection, but if the virus has a wide host range the dangers of such widespread, and often unrecognised, infection-foci are obvious. Thus the use of carrier-varieties may solve a disease problem for one particular crop only to raise a problem for another. Carrier-varieties have a further disadvantage, which is well illustrated by the potato. All American commercial varieties seem to be 100% infected with virus "X" and most stocks of British varieties which tolerate infection have a high percentage of infected plants. Whether the presence of this virus affects the cropping power significantly when it causes either a mild mosaic or no definite symptoms has not been shown conclusively, although SCOTT (1941) and BALD and NORRIS (1940) consider reductions of 20% common. But when such stocks become infected with an additional virus, such as virus "A", which alone has little or no effect, they become seriously diseased. Also, a potato variety may tolerate one strain of virus "X" but be severely affected by another strain. As mutation in viruses seems to be fairly common the possibilities of varieties ceasing to be carriers must also be considerable.

Because of the difficulties of keeping tolerant varieties virus-free, considerable attention is now being given to the raising of extremely intolerant varieties. Paradoxically enough this promises to be of considerable value in controlling some diseases, as it can convey what amounts to the virtual immunity of field crops in one of two ways. Varieties may be so intolerant of infection that their tissues die quickly on contact with the virus, so that the pathogens become isolated in

pieces of dead tissue around the entry points. HOLMES (1938) has found that the ability of *Nicotiana glutinosa* to localise infection with tobacco mosaic virus is due to a single dominant gene. He has transferred this gene to *N. Tabacum* through the species *N. digluta*, and by repeated backcrosses has obtained tobacco-like derivatives with the ability to localise infection. The incorporation of this gene into tobacco varieties of a high quality should do much to reduce the losses now caused by tobacco mosaic virus. Not only should it greatly reduce the amount of infection in the tobacco crop itself, but it should also help to free other crops such as the tomato, which now often become infected from contaminated tobacco. CLAYTON and MCKINNEY (1941), however, throw some doubt on the practical value of this type of resistance. They state that in the field, especially in the major tobacco-producing areas where the summer temperatures are high, infection is often not restricted to a local necrotic reaction. Instead, inoculated plants develop a systemic necrotic disease which is often fatal. Even so, this in itself should prove a considerable help in limiting spread, for it is the second type of hypersensitivity being sought by plant breeders with other crops. Such seriously affected plants are soon observed and can be removed before they have the opportunity to act as sources of further infection. Even if they are not removed, their early death makes them of little importance as sources of spread compared with normal mosaic-affected plants, which remain with a high virus-content throughout the growing season. Varieties which die when infected, in effect do their own roguing. The loss of the infected individual is complete, but the loss to the crop is negligible and transitory. Several potato varieties widely grown in the British Isles are killed by infection with one or other of potato viruses "A", "B", "C" and "X". Commercial stocks are rarely found contaminated with a virus which kills that particular variety, whereas the more tolerant a variety is to a virus the more are the commercial stocks likely to be infected. By crossing between various varieties, the Scottish Society for Research in Plant Breeding (BLACK 1939) has produced Craig's Defiance which dies with top-necrosis when infected with any of the four viruses. Unfortunately, no parents are known which are so intolerant of potato virus "Y" and leaf roll and the chances of producing varieties that will remain free from these is less hopeful. However, some varieties become infected much more slowly in the field than others, although they take the viruses readily and react clearly when infected experimentally. The reasons for this slower rate of infection in the field are not known, but they may be connected with variations in the ease with which insect vectors can infect different varieties. SCHULTZ *et al.* (1940) have shown that varieties of potato susceptible to virus "A" can be divided into those which never become infected in the field, those which are rarely infected and those which are readily infected. They find that some varieties which are susceptible to this virus by grafting are immune from infection by aphids.

CLAYTON and MCKINNEY (1941) consider that the type of resistance to tobacco mosaic shown by the variety Ambalena (NOLLA and ROQUE 1933) and T.I. 448 (CLAYTON *et al.* 1938; MCKINNEY 1939) is more

likely to be of practical value than the necrotic reaction. This type of resistance depends on two recessive pairs of genes and has been maintained through numerous backcrosses. In the main it seems to take the form of tolerance towards infection, coupled with a smaller rate of multiplication and movement of the virus than in ordinary tobacco, rather than a true resistance to infection.

Although there are obviously great opportunities for the production of varieties of crops plants that will suffer less from virus diseases than those now in commerce, it is improbable that this alone will give a permanent control of all virus diseases. One of the difficulties in this type of control is the uncertainty in forecasting to what extent a character found to be desirable on an experimental scale will be retained in nature. The ability to carry a virus, to localise infection or to show a clearly defined syndrome, may be greatly affected by environmental conditions. Also, plants may react very differently to different strains of the same virus, and many viruses seem to mutate frequently to give new strains that cause different syndromes and have different host ranges. It is, therefore, impossible to be sure for how long, and in what conditions, new varieties which are resistant, tolerant or possess some other desirable feature, will retain this property in cultivation.

Since it has been found that plants infected with one strain of a virus are protected against other strains, and strains of some viruses, for example, tobacco mosaic (HOLMES 1934) and potato virus "X" (SALAMAN 1938), have been derived that produce no apparent effect on some hosts, the possibility of controlling the losses caused by virulent strains by a process of vaccination with avirulent strains suggests itself. SALAMAN points out that this process has actually been going on in nature in the potato, for many of the oldest and strongest varieties are fully infected with mild strains of potato virus "X" and so protected against the severe diseases produced by other strains. As far as the writer is aware no large scale tests of this method of protection have yet been attempted. Experimental plots of tomatoes infected with a masked strain of tobacco mosaic virus were found to yield better than either plants infected with a virulent strain of tobacco mosaic virus or those not deliberately infected. Insufficient work has yet been done to decide whether or not this method will prove of any value. The practical problems of infecting plants with avirulent strains could no doubt be overcome without much difficulty. For example, with crops such as tobacco and tomato, which are transplanted as seedlings, if the hands of planters were covered with sap from plants infected with avirulent strains, infection would occur during planting; and with crops like the potato reproducing vegetatively, it would be necessary only to infect plants whose progeny was destined for use as seed. There are, however, serious objections to the use of the method until it has been thoroughly tested, for it is accompanied by far greater dangers than the vaccination of animals, which it superficially resembles.

In Chapter 6 it was shown that the exact mechanism underlying the acquired immunity is not known, but is of the non-sterile type, plants being protected against other strains of a virus only while they are

fully infected with one. The vaccinated plants would, therefore, always contain active virus. Virulence is often a virus-host relationship and viruses which produce no, or very slight, symptoms on one plant may produce severe diseases in others; for example, some viruses and virus strains avirulent towards tobacco and some potato varieties, cripple or even kill other potato varieties. As there is also good evidence that some avirulent strains can mutate to give rise to virulent ones, it would seem a dangerous policy to distribute viruses widely as vaccines, even when they have been found to be avirulent experimentally.

The vaccination of plants by inoculation with apparently avirulent viruses presents another, and perhaps even more serious, disadvantage, for the protection is limited to related strains of the vaccine. There is the obvious danger of the vaccinated plants becoming infected with an unrelated virus, and such a double infection often results in a much more severe disease than the second virus would cause alone. Potato virus "A" in many potato varieties causes only a transient mottle, but if the plants contain virus "X" it will produce crinkle. Similarly, potato virus "X" usually has little serious effect on tomatoes, but if these were "protected" by infection with masked tobacco mosaic virus it would cause a serious necrotic disease. Because of these interactions, it is obviously impracticable to vaccinate plants against more than one virus. The application of the method, therefore, would seem to be restricted to particular districts in which plants are severely affected by virulent strains of one specific virus.

Insect-transmitted viruses:—Three conditions must be fulfilled before insect-transmitted diseases can spread. There must be a source of infection, the insect vector must be present and it must be moving about from plant to plant. Control measures, therefore, can be designed to remove sources of infection, to eradicate the vectors or to inhibit their movements. Roguing within the crop is again of value, but with viruses whose vectors are continually on the wing it can only be effective if practiced at frequent intervals, for fresh infection can be continually introduced by the arrival of infective insects.

As different insects are transmitted by insects having different habits and life histories, accurate diagnosis of the causative virus is needed before any control measures can be adopted, but most vectors are insects with sucking mouthparts so that only fumigation or contact insecticides will usually be of value. The problem of controlling insect vectors is not strictly comparable with the control of insects acting directly as pests. For the latter, a total kill is not necessary, as the insect population merely needs reducing below the level at which feeding alone causes appreciable losses. For successful control of vectors, however, more than this is needed, as considerable spread of viruses can occur with far too few insects to constitute a pest. Continued and frequent sprays or fumigations will therefore be needed to achieve results, and for the control of aphids and leafhoppers in crops grown on a large scale this is probably not economic. Where potatoes are regularly sprayed or dusted against *Phytophthora infestans*, however, the addition of an insecticide might more than justify the expense.

Only a few attempts to control virus diseases in the field by combatting the vectors have yet been made, but these suggest that they may be practical, at least with crops grown on a small scale and intensively cultivated. WATSON (1937), for example, found that significantly larger yields of *Hyoscyamus niger*, justifying the cost, were obtained by weekly sprayings during June and July with nicotine against the aphids that spread potato virus "Y" and *Hyoscyamus* virus 3. MOORE (1941) has also reduced infection with spotted wilt virus in tomato crops in South Africa from 100% to 5% by spraying with tartar emetic. Diseases such as peach yellows, which is spread by an insect having only one brood a year and active for only short periods, and yellow edge and crinkle of strawberry, whose vectors have only a limited migratory period, could almost certainly be reduced by the use of insecticides at the appropriate times. Considerable success has been achieved in controlling cranberry false-blossom by flooding the fields, or by spraying the plants, to eliminate *Euscelis striatulus* when they first hatch out in the spring (WILCOX and BECKWITH 1935). The virus causing pupation disease of oats overwinters in the hedgerows and headlands, either in grasses or in the larvae of *Delphax striatella*. By spraying against these in the winter, SUKHOV and PETLYUK (1940) have greatly reduced the severity of attack.

In glasshouses fumigation at regular intervals is usually sufficient in preventing insect-transmitted diseases. For the control of spotted wilt virus, however, other precautions should also be taken. At the end of each tomato crop, glasshouses should be emptied and then thoroughly fumigated with cyanide. One of the commonest rotations in glasshouses is tomatoes and chrysanthemums, both of which are hosts for thrips and susceptible to spotted wilt virus. As the first chrysanthemums are often brought in before the last tomatoes are out, any infective thrips can migrate to the new plants and so perpetuate the disease. This virus has an enormous host range and all weeds should be removed and burnt before the house is fumigated.

The time of planting can sometimes play an important part in determining the severity of attack. WALLACE and MURPHY (1938) have shown that early planting of sugar beet greatly reduces the losses caused by curly top, for plants are well grown before the arrival of the leaf-hoppers and the disease is only crippling to plants that are infected in the seedling stage. WATSON (1942) has similarly shown that early planting greatly reduces the loss in yield and sugar content produced by sugar beet yellows virus. The sugar beet seed crop is an important over-wintering source of sugar beet viruses and wherever possible this should be isolated from the main sugar crop. WALLACE and MURPHY (1938) have also demonstrated the value of removing weeds from the vicinity of sugar beet crops, for many species can act as winter hosts of both curly top virus and its vector. Surrounding crops with a barrier to prevent or lessen the entry of insects has been successfully used. In the U. S. A. large numbers of asters are now grown under tents, or behind screens, made of muslin with a sufficiently fine mesh to prevent the entry of leafhoppers, since this has been found the only effective method of controlling aster yellows. In Maine this method has also been found to be the only reliable one for

keeping selected potato seed quite free from virus diseases. FOLSOM (1942) reported that although when this was started it was not expected to be commercially successful, the cage is profitable because it so greatly reduces the cost of roguing. SUKHOV and PETLYUK (1940) found that oat fields surrounded with a gauze fence 2 metres high escaped pupation disease, and they suggest that living hedges afford considerable protection against swarms of winged insects.

The virus diseases of the potato are controlled in the main by growing the seed crops in conditions that inhibit the multiplication and movements of aphid vectors. MALDWYN DAVIES (1935, 1936) has shown that these are temperature below 65° F, relative humidity over 70, and a wind velocity of over 8 m.p.h. and absence of sunlight. In the British Isles the weather in the north and west supplies most of these conditions, and long before virus diseases were recognised it was customary for potato growers in the south and east to obtain new seed from these districts. With the recognition that potatoes degenerated because of virus diseases, further steps have been taken to improve on the natural advantages of the seed-growing districts. Crops are rogued at frequent intervals, and they are inspected during the growing season and awarded certificates of health guaranteeing that they contain less than a small percentage of infected plants. The growers of ware potatoes in the warmer and drier parts of the country, where aphids are abundant and continually on the move, regularly buy new stocks of this certified seed. The rate at which this seed degenerates varies with the season, the district and the conditions under which it is planted. Usually, not more than about 10% of the seed saved from such certified stocks in the south and east of England is virus infected, and the seed-sized tubers from these crops are planted again. In the second season the infection usually reaches 50% or higher and the stock becomes useless for seed purposes. Thus growers of ware potatoes usually save seed from their own stocks only once.

In other countries there is a similar transfer of seed potatoes from districts in which spread of virus diseases is less rapid, usually near the coast or on high ground, to the main potato growing areas. In the U. S. A., in addition to certification, further precautions are taken in the selection of seed. The most important are the tuber-indexing method and the tuber-unit method (DYKSTRA 1941), of which the first is the more effective. In the tuber-indexing method all seed tubers are numbered and an eye is taken from each and planted well in advance of the normal planting in the field. In the Southern States, where two crops can be taken in a year, the planting is done in the field, but in the Northern States it is usually done in glasshouses or the tuber-indices are sent to the South for planting. If the shoot that develops from an eye shows any sign of disease, then the tuber from which it was taken is discarded. Certification of growing crops is not entirely a reliable method of ensuring virus-free seed, for symptoms, especially of leaf roll, often do not show in the first year of infection, and sometimes a high percentage of plants may pass unnoticed if there has been a late infestation with aphids. As a check on field certification, therefore, some American States and certification agencies use the tuber-index method on samples from their stocks to get a true reading

of the amount of virus infection at the time of lifting. In the tuber-unit method, cut tubers are used and the pieces from one tuber are planted in units separate from others. This facilitates examination and roguing, and if the plant from one seed piece shows any symptoms all the others from that tuber are dug up and destroyed.

The principle of separating the potato seed growing districts from those producing ware potatoes is effective in reducing losses, but it has disadvantages. Firstly, certified seed is expensive and the costs of transport over large distances are high. The seed often arrives too late to be chitted before planting and there are often considerable losses from damage in transit. By adopting a few precautionary measures for the production of their own seed, it is probable that growers, even in districts where degeneration is normally rapid, could keep their stocks for much longer than they now do. The method generally used for the selection of seed potatoes is so bad that it might almost have been expressly designed for the increase of virus diseases. The product from a whole crop is passed through riddles of various sizes, the largest potatoes being sold as ware for eating and the smallest being retained for seed. As it is one of the commonest effects of virus infection to reduce the size of tubers, this practice leads directly to an increase in the percentage of infected plants in succeeding crops. It is also a common practice for growers to buy sufficient new seed each year to plant half the required acreage, the other half being planted with seed saved from the preceding year's crop. In this way the new seed is immediately exposed to infection and it is not surprising that it degenerates rapidly. If, when buying new seed, sufficient were purchased to plant the whole acreage, this rapid infiltration of virus from nearby infected potatoes would be largely eliminated. Again, instead of planting all the crop together, if about an eighth were planted away from the rest, preferably separated from other potatoes by a corn crop, to serve as seed in the next year, the health of the stock could probably be kept at a reasonably high level for several years longer than by present methods. Such small plots could be inspected at frequent intervals and all suspicious plants removed; they could also be kept free from weeds and groundkeepers, and sprayed regularly against aphids, a practical impossibility with the large fields now grown primarily for ware. To reduce the chances of infection, the period of growth could be reduced by removing the haulm 6 to 8 weeks before the crop is mature. This treatment does not reduce the number of tubers set by the plants, but only their size. Different potato varieties may show quite dissimilar clinical syndromes when infected with the same virus, and apparently healthy individuals of one may be infected with a virus lethal to other varieties. It is a wise precaution, therefore, not to grow different varieties near to one another, for one may be an unrecognised source of danger to the other.

The most important vector of potato viruses is the aphid *Myzus persicae*. The egg stage on peach and apricot trees is the normal method of overwintering, but viviparous insects also overwinter in glasshouses and, in temperate conditions, on brassicae crops such as turnips and Brussels sprouts (MALDWYN DAVIES 1935, 1936). From the winter hosts the winged forms migrate in the spring, usually first

settling on cruciferous weeds, then passing to potatoes when these come above ground. The first potatoes to appear are groundkeepers from the previous year's crop. In districts where virus diseases spread rapidly, these are likely to be diseased, so that the aphids can be already infective by the time they reach the main crop. Whether treating the winter hosts to eradicate the vector, for example by fumigating glasshouses and winter-washing peach and apricot trees, would be a practical method of control is unknown. But, wherever possible, crops of potatoes planted for seed should not be grown near the winter hosts and groundkeepers and cruciferous weeds in or near the crops should be removed.

Mechanically-transmitted viruses: — The viruses to be considered in this section are those of the tobacco mosaic type whose peculiar properties call for special control methods. As far as is known insects play no part in the transmission of these viruses and they owe their widespread distribution to the ease with which they are transmitted mechanically and to their great stability. The viruses reach a high concentration in their hosts and the handling or cutting of healthy plants after handling or cutting diseased ones, or the rubbing together of healthy and diseased leaves, is sufficient to produce transmission. Thus, once a few plants in a crop have become infected, ordinary cultural operations can soon lead to widespread infection. The viruses are so stable that they resist drying and can remain active in diseased plant material for many years. It is from such material that initial infections arise, and control methods depend on the elimination of these primary infections and on modifying cultural practices so that the amount of spread is reduced. In this section control in the tobacco crop is mainly considered, but similar measures are also effective in the tomato crop.

One of the most common sources of initial infection is from the hands of workers who use tobacco either for smoking or chewing. VALLEAU and JOHNSON (1937) and BERKELEY (1942) have shown that a high percentage of infection arises, especially during the transplanting of seedlings, or weeding the seed-bed, unless precautions are taken to ensure that the workers' hands are not contaminated. The use of barn-cured tobacco is especially dangerous, as this is usually highly infective, but even commercially prepared tobacco is also often a source of initial infections. During cultural operations, therefore, workers should not use tobacco in any form, and before starting work they should wash their hands thoroughly with soap and water.

The second main source of primary infection is from contaminated roots or leaf debris in the soil, in which the virus can remain active for periods of years (JOHNSON 1937). Infection rarely, if ever, seems to occur through the roots, but through wounds in the leaves and stems produced by cultural operations. BERKELEY (1942) found that there was little infection from contaminated soil in seed beds if the plants were left undisturbed but there was much when weeding was done. He emphasises the necessity of efficient soil sterilisation where a permanent seed-bed is employed and of excluding the possibility of infections from outside sources, for the presence of a few infected plants

in the seed-bed gave 75% infection in the field crop as a result of the handling during transplanting. VAN SCHREVEN (1941b) found that the addition of tannic acid to the soil prevented infections from this source, and treatment of seed beds, tools, boxes, etc. with this may be a practical control measure. In the field, the problem of eliminating infection from the soil is more difficult. The residues from infected crops should not be ploughed in and tobacco trash should not be used as a manure, but the roots are not easily removed. JOHNSON (1937) has shown that the soil can only be freed from contamination by crop rotations in which non-susceptible plants are used and by frequent cultivations to expose the soil to weathering conditions that cause inactivation. Seed should only be taken from virus-free plants, for although the virus is not seed-transmitted in the ordinary sense and does not give rise to infected plants directly, AINSWORTH (1933) has found that the virus is often present in the testa and so forms a further possible source of primary infection in the seed bed.

When a high percentage of infection occurs early in the crop, rogueing is unlikely to produce any satisfactory results, but where the field crop contains only a few infected plants these should be removed before any cultural operations are started. When tobacco plants are being topped, or tomato plants tied or dis-budded, healthy plants should be treated first, then those near diseased plants and the diseased plants themselves left until last. Cultural operations should when possible be carried out in fine weather, for BERKELEY (1942) found that more than four times as many plants became infected after cultivating when the plants were wet with rain or dew as after cultivating when the sun was shining. Periodic disinfection of hands and implements by dipping in solutions of Na_2PO_4 has been found effective in reducing spread (VAN DER WEIJ 1940), but such a strong alkali is likely to have deleterious effects on the skin if used repeatedly. VAN SCHREVEN (1941a) found that 0.5% solutions of commercial tanning substances were as efficient as the phosphate, and it is likely that some of the substances described in Chapter 11, which act as inhibitors of infectivity, would also be of value. Repeated dipping in sap from *Phytolacca decandra*, milk or whey, so that hands and implements were always wet with these, would probably greatly reduce transmission without producing any deleterious effects.

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DISCUSSION ON THE ORIGIN AND MULTIPLICATION OF VIRUSES

Although for practical purposes it is of little importance whether viruses are considered as living or non-living, few questions have aroused such general interest as this. It has continually been discussed without any unanimous conclusion being reached, though individuals have often expressed their own conclusions in no uncertain manner. Viruses were discovered at about the same time as the fermentation of sugar and some other processes, previously regarded as "vital", were shown to proceed in the absence of living cells, and the controversy over viruses arose in place of the earlier one on the reactions now known to be caused by enzymes. Until the last few years, most workers have been content to adopt the view that viruses are sub-microscopic, living organisms. With the discovery that some plant viruses can be obtained in the form of crystalline or liquid crystalline protein preparations, however, this view has been less widely held, and phrases such as "lifeless molecules" have increasingly been applied to them. The truth is that the question as such is unanswerable. The difficulty in providing an answer in the past was generally assumed to be ignorance of the nature of viruses, and it was believed that once the constitution of a virus was known, the problem would be solved. But now that it has been shown with reasonable certainty that many plant viruses are nucleoproteins, the question still remains unanswered. The present difficulty lies less in our ignorance of viruses than in deciding exactly what the question itself means.

PIRIE (1937) has clearly pointed out that life and living have no rigidly definable scientific meanings and that, until they have, it is impossible to draw a clear-cut division between living and non-living systems. Everyone has an aesthetic appreciation of the manifestations of life, and the words have a fairly definite meaning when applied to such diverse systems as dogs and kennels or moss and stones. There is, however, no single criterion of life; the phenomena associated with it, such as reproduction, growth, respiration and movement can all be simulated by systems which would not usually be considered alive. There are many systems showing intermediate degrees of organisation which are often neither obviously dead nor obviously alive. For example, the dog as a whole will be accepted by everyone as alive*, but if all the individual components which go to make up the dog are examined separately it will be impossible to decide whether many of them should be regarded as living or as non-living. Again, tests for living which are accepted as decisive for one system are often not accepted for others. A castrated dog, for instance, would still be

* Unless obviously dead!

regarded as living although it has lost the power of reproduction, often considered the most characteristic property of life. On the other hand, bacteria treated in such a manner that they have become incapable of reproduction would certainly be regarded by most bacteriologists as dead.

At the level of organisation exhibited by viruses it is not difficult to argue at great length both for and against the view that they are living, for it is obvious that viruses possess some properties usually considered characteristic of living systems and others more often associated with non-living systems. Until the word life has been given an accurate definition, however, which definition may well come from further studies on viruses, the argument is unlikely to be concluded. Whether individual workers conclude that viruses are living depends greatly upon their own conception of the most important manifestation of life. Their conclusion is also influenced by the particular virus with which they have worked and by the medium in which their investigations have been conducted. When studied in infected plants or animals, viruses exhibit few phenomena conflicting with the view that they are small organisms. They multiply readily, frequently mutate, and can often be adapted to new hosts, properties usually only associated with living systems. *In vitro*, on the other hand, they appear to be quite inert, their behaviour approximating much more closely to that of protein molecules. Their small size, obligate parasitism, and properties *in vitro* are most often advanced as evidence that viruses are non-living. None of these, however, would seem to be conclusive.

The existing evidence on the sizes of viruses suggests that they range from around the limit of microscopic resolution down to particles of about 20 m μ in diameter or even smaller. The larger viruses, such as psittacosis and vaccinia, have particles as large as the bodies of some accepted organisms which multiply readily and metabolise *in vitro*, such as those isolated from sewage by LAIDLAW and ELFORD (1936) and those causing bovine pleuropneumonia. At the other end of the scale, the particles of tomato bushy stunt and foot-and-mouth viruses are no larger than the molecules of the protein haemocyanin, generally accepted as non-living. Between these extremes virus particles of all intermediate sizes have been described. Unless, therefore, it is to be assumed that the agents grouped together as viruses are a heterogeneous collection, no conclusions can legitimately be made on the basis of size. It has often been stated that particles of the size of the smaller viruses are too small to contain the complexes thought to be necessary for organised life. But such a statement is obviously valueless unless it is accompanied by a second defining these complexes and the size of the smallest particles able to contain them. This second statement, of course, cannot be made, for if it could it would supply the required definition of life in terms of size or complexity, and there would be little difficulty in stating whether viruses should be considered as alive. Also, although small relative to bacteria, even the smallest virus particle could contain large numbers of proteins with the more usual molecular weights of from 30,000 to 100,000.

It is not known whether viruses have any independent metabolism when multiplying in infected tissues. The highly purified preparations

seem to have none, those of tobacco mosaic virus and tomato bushy stunt remaining unchanged over long periods. At first sight this seems strong evidence that they are not living. However, the apparent absence of metabolism *in vitro* does not necessarily imply an essential difference from accepted organisms, for some of these when placed in unfavourable environments enter into resting stages as spores, seeds or eggs, during which states often no respiration can be detected. That no viruses have been cultivated *in vitro* is a further fact frequently advanced as evidence that they are not living and that they do not reproduce themselves, but are produced by their hosts. There is no valid reason why this argument should be restricted to sub-microscopic pathogens; if pushed to its logical conclusion it would become necessary to assume that all the rusts as well as the other fungi and bacteria that have not yet been cultured artificially are also non-living. There are, however, probably few pathologists who would be prepared to accept obligate parasitism as evidence that these organisms do not reproduce themselves.

Many writers appear to find an essential incompatibility between the living and the crystalline states, and because viruses can form crystals or liquid crystals they automatically conclude that viruses are not living. But again there seems to be no scientific basis for this view. It is true that no accepted organisms have been made to form crystals, but this may merely be a result of their large sizes. There is no immediately obvious reason why particles possessing many of the attributes of organisms, if sufficiently small and even in size, should not arrange themselves with a three-dimensional regularity. Crystallinity simply implies a regular arrangement of particles and such orderly arrangements are commonly encountered in nature. Plant fibres, hair, muscle and fish sperm are all birefringent and hence have their constituents arranged with some degree of regularity. Birefringence has also been noticed frequently in nuclei undergoing division. Any collection of rods of equal cross-section must of necessity become orientated when made to flow or when packed tightly, and BAWDEN and PRUE (1937) have pointed out that the orderly arrangement in the purified preparations of viruses showing anisotropy of flow differs in no essential manner from that in suspensions of rod-shaped bacteria or even in shoals of fish. It would be decidedly unwise to assume that the units in an orderly structure are not living merely because the word crystalline can be applied to them.

Although it is probably unprofitable to discuss further at the present time whether viruses are living, it is of value to compare them with recognised organisms. The difficulty in deciding if viruses are living is largely the difficulty mentioned earlier in deciding exactly which components of any accepted living system should on their own be regarded as living. For the purified viruses differ quite clearly from organisms, and in many ways resemble constituents of organisms rather than the organisms themselves. All those that have yet been obtained in a state there is any reason to believe to be pure have been found to consist solely of nucleoprotein. They may, of course, contain many different kinds of protein molecules, but the only constituents isolated from them after disruption by many different methods have

been protein and nucleic acid. This chemical simplicity contrasts remarkably with the complexity of the simplest recognised organisms which, in addition to different kinds of protein, contain a variety of other substances both diffusible and indiffusible. Even this difference, however, may be less real than it appears. The nucleoproteins must be regarded as the viruses because of their infectivity and ability to multiply, but as any change in them results in loss of infectivity they can only be regarded as the simplest form in which the viruses can exist. When multiplying in their hosts it is possible that they are chemically more complex, and some of the so-called impurities removed during the purification processes may not be normal plant products but products of virus activity. If, as seems probable, viruses have no limiting membrane equivalent to a cell wall and their activities are largely at the surface, then any of their products would be separable from the nucleoproteins and would not be held together as a recognisable whole as in a bacterium. Products of virus activity and constituents of the infected host plant cells thus would normally be indistinguishable.

There is no evidence for this view with plant viruses, except that some of the impurities are extremely difficult to remove from potato virus "X" and tobacco mosaic virus, and their removal seems to be accompanied by changes in virus properties. From the relatively large elementary bodies of vaccinia, however, a specific, immunologically active carbohydrate can be liberated (HUGHES, PARKER and RIVERS 1935; SALAMAN 1937). This is not found in healthy animals but is always associated with vaccinia infection, and it can be removed from the elementary bodies without in any way affecting their infectivity. It can hardly be doubted that the carbohydrate is a part of, or at least produced by, the virus, although it is not a constituent essential for infectivity. In the same way it is possible that there are other inessential, removable, constituents of this and of other viruses, but that these have not been identified with the viruses because there is no specific test for them. The serological reactions provide such a test for the soluble antigen of vaccinia.

Other significant differences between the purified plant viruses and organisms are indicated by the X-ray patterns. These show that in addition to the regular arrangement of the particles to form crystals or liquid crystals, the constituent groupings making up the particles are arranged in the perfect regularity of a crystalline lattice (Fig. 48). Each particle has an internal regularity of the type sometimes found in large molecules; but with the viruses it is on an unusually large scale. In this sense, the virus particles resemble animal or plant fibres more than organisms. Again, most organisms contain large quantities of water, but the interior of the virus particles is relatively free from water even when they are in solution. This is shown by the fact that the spacings between the constituent groupings of the virus particles are but slightly different whether the X-ray measurements are made on solutions or on films of completely dried virus.

As the sizes of virus particles cover the range between organisms and molecules, it is often suggested that viruses are a collection of heterogeneous agents, the largest being parasitic organisms similar to

bacteria and the smallest being inanimate protein molecules. Such a view, however, ignores the fact that viruses of all sizes have many properties in common and that there is a steady gradation in size from the largest to the smallest. Those who make the suggestion rarely attempt to classify the viruses with particles of sizes interme-

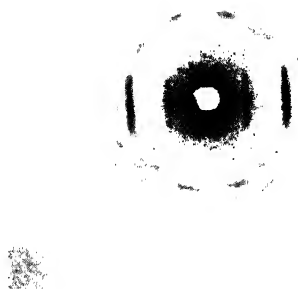


FIG. 48. — X-ray photograph made on a 15% solution of tomato enation mosaic virus, showing the pattern obtained by scattering of X-rays at wide angles. These spacings are almost independent of the solid content of the preparation, and the pattern results from regularities of arrangement within the virus particles. (Photograph by I. FANKUCHEN. Specimen 8 cm. from a copper anticathode, nickel filter, exposure 170 hours).

diate between the largest and smallest. Neither do they give any indication as to the size at which viruses are to be regarded as ceasing to be organisms and to become protein molecules.

To explain the range in size of virus particles the view has been put forward that viruses represent a transition stage between molecules and organisms, representing a sort of half-way stage between animate and inanimate systems. Some writers consider that they are an upward stage in evolution, suggesting that they resemble the form in which "life" first originated, and others that they have been produced by a retrograde evolution from larger organisms. Until large numbers of saprophytic viruses have been discovered the first view is barely worth discussing, for obligate parasitism, one of the most characteristic features of recognised viruses, is hardly a property favouring the continuation of the first forms of "life". The second is much more plausible. It is well known that some parasitic bacteria can grow in culture

only if the medium contains some substances fully formed, although other related bacteria have the power of synthesising these. It is thought that the bacteria have lost the ability to synthesise all the compounds necessary for growth because they have continually existed in an environment where the compounds are provided fully formed by the host. GREEN (1935) and LAIDLAW (1938) have adapted this theory of a loss of function and form produced by continued parasitism to explain the origin of viruses from organisms like bacteria. It is suggested that viruses were originally all organisms, which have come to rely on their host's metabolism for more and more of the substances essential for multiplication and growth. With this increasing dependence upon the host it is believed that the organisms have lost the ferments necessary for synthesising essential growth substances. LAIDLAW suggests that the largest viruses are only slightly changed organisms, having lost only a few ferments, the intermediate-sized viruses are supposed to have lost several, and the smallest to have lost all activities except those necessary for the propagation of the species. This theory cannot at present be proved or disproved. If true, however, it might be expected that the larger viruses would be chemically much more complex than the smaller. For instance, they might still be expected to retain the cell wall of their parent organisms. With a knowledge of the postulated growth factors they should also be easier to grow *in vitro*. The assumption that the inability to synthesise all the requirements for multiplication is of necessity evidence of retrograde evolution and of a loss of size, however, raises wide implications, for it would become necessary to assume that all organisms except autotrophic bacteria and green plants, which are the only ones capable of synthesising all their growth requirements, have evolved in this manner from larger organisms.

In its ultimate result the theory of the production of viruses by the simplification and decrease in size of larger micro-organisms approximates to the older "free gene" theory. MULLER (1922) and DUGGAR and ARMSTRONG (1923) pointed out that viruses and genes both multiplied only in living hosts and that they often produced somewhat similar results. They suggested that viruses might be genes which had escaped from the nucleus and were multiplying independently of it. Since some plant viruses have been found to be nucleoproteins this theory has been revived, for genes are also often assumed to be nucleoproteins. This assumption is made because the chromosomes are rich in nucleoprotein, but it is by no means necessarily valid. It is possible, for example, that the genes are merely small active groups carried on the chromatin basis. Again, this theory of the origin of viruses cannot be disproved, but there is some evidence against it. A gene is usually regarded as a single inheritable factor, but there is now considerable evidence that viruses can contain a number of such factors. HOLMES (1936) found that the change over in tobacco mosaic virus from an invasive to a non-invasive type was quite independent of the change from a form causing a yellow mottle to one causing a green mottle. NORVAL (1938), although he likens this virus to a "free gene", also claims to have detected several inherited factors for the causing of necrosis. Similarly, in potato virus "X", SALAMAN (1938) describes

five factors responsible for different symptom production. It would seem then more reasonable to compare the virus particles with groups of genes or with fragments of chromosomes rather than with single free genes. It is, of course, possible that viruses and chromatin reproduce in the same manner and produce their characteristic effects by the action of specific side groups. The chemical evidence, however, does not support the view that viruses are nuclear fragments. The only nucleoprotein that has yet been demonstrated in nuclei contains thymus nucleic acid. By contrast, the nucleic acid in all the viruses yet purified has been found to be of the yeast type, containing a ribose instead of a desoxy-pentose. There is no simple test for a nucleic acid of the ribose type, so that it is possible that nuclei contain this in addition to thymus nucleic acid. It is also possible that bacteria, in which normal nuclear material has not clearly been demonstrated, may contain ribose-type nucleoprotein as a bearer of hereditary factors. But until experimental data have been obtained supporting this, there would seem to be little value in comparing viruses with genes.

As viruses in their simplest forms can be obtained as nucleoproteins, a theory of the origin of viruses as equally probable as any others put forward is that they have arisen as a result of "accidents" in the protein metabolism of organisms. The accidents may have occurred in the organisms which are now affected by the viruses or in others in which they may possibly have no adverse effects. Indeed, it is not beyond the bounds of possibility that the viruses now known as causing diseases in some hosts may be invariable constituents of other organisms. The absence of any proteins similar to the viruses in the control healthy plants of the species yet investigated may render this unlikely, but there is one example that at least superficially appears to support it.

SALAMAN and LE PELLEY (1930) have found that every King Edward potato plant examined, however healthy in appearance, is actually virus-infected. Some hundreds of individual plants from different districts have been examined by these and other workers and every one has been found to cause a crinkle when grafted on to Arran Victory and some other potato varieties. This virus has not been transmitted by mechanical inoculation methods, nor has any insect vector been discovered for it. In spite of the widespread occurrence of this virus in the much grown King Edward variety, as far as the writer is aware this virus has not been found naturally in any other potato variety or in any other plant species, although these are quite susceptible when artificially infected, some behaving like King Edward and showing no symptoms and others reacting with symptoms of varying degrees of severity. Here, then, is a constituent of every King Edward potato plant that can be transferred to other plants in which it multiplies and may cause severe diseases. This constituent thus has all the necessary qualifications to be called a virus. Unfortunately no work has been done on its chemical nature, but as all the other viruses isolated have been so alike, and some of these have been obtained from hosts showing no symptoms, it is not unreasonable to assume that it will also be a protein. It is, of course, possible that the original King Edward plant became infected with a virus from an external

source and that this virus has been transmitted unchanged through all the progeny. But no similar virus has been found in other plants and the virus in King Edward potatoes is transmitted only by grafting. If this view be accepted, it seems necessary to assume that the virus on entering the original King Edward plant suffered such a change in properties that it can no longer be transmitted by the method whereby it entered the plant, or be related to the more readily transmissible virus from which it has mutated. It is at least equally plausible that this virus is a normal constituent of King Edward plants, a protein produced in the course of metabolism with the property of reproducing itself in this and other plants. The accidental production of a new protein of this type will probably be a rare event. The King Edward potato is the only example known to the writer of a plant invariably containing a virus that has not been recorded elsewhere. Such occurrences may be less rare than they appear, for a virus arising in a plant in which it produced no abnormal symptoms would usually remain undetected. It would become apparent only if transmitted to another plant in which it could not only reproduce but also produce symptoms. This normally could happen only with viruses transmitted by insects or by mechanical means. Many viruses may have arisen only to disappear with the death of their host plant. The detection of the virus in King Edward resulted solely from the unusual and artificial procedure of grafting portions of this plant to other potato varieties, and its persistence can be attributed solely to the method of propagating potatoes. Should such a virus arise in a plant not reproduced vegetatively it would disappear with the death of that plant. Similarly, should a virus cause a lethal disease in the host in which it arises it would again probably disappear with the death of the parent plant. Only those viruses causing less severe diseases, or no symptoms, and which are transmitted efficiently by some method, either mechanically, by insects or by continued vegetative propagation of the original host, would be expected to survive and become at all widely distributed. These, of course, are the properties of the plant viruses found commonly in nature. Although this theory of the origin of viruses within their hosts by occasional accidents in the protein metabolism would conveniently explain many other at present inexplicable phenomena, it must be emphasised that it is merely speculative with no supporting experimental data.

A discussion of the methods whereby viruses multiply in their hosts can similarly only be speculation, for again there is little experimental work with a bearing on the problem. Structurally, viruses differ so strikingly from organisms that it is perhaps improbable that they multiply in a similar manner by binary fission. Binary fission, however, can only be regarded as the last step in a process whereby one organism, or one cell, becomes two. Both before and after fission there must be multiplication of the constituents of the organism or cell, and it is probable that the multiplication of viruses is more comparable with the multiplication of these than with the multiplication of the organism itself.

The multiplication of tobacco mosaic virus is independent of photosynthesis, for it occurs readily in excised roots and in cut leaves

kept in the dark. The rate of multiplication is greatly increased by increasing the temperature at which infected plants are kept and the extent of multiplication depends on the nutrition of the host. SPENCER (1941*a* and *b*) grew infected plants in normal and in nitrogen-deficient conditions and found that both the virus and the soluble host-protein remained constant in the deficient plants whereas it increased more than five times in 16 days in the plants receiving nitrogen. Similarly, when young plants were infected by rubbing over their whole leaf surfaces, multiplication was more rapid in those getting ample nitrogen. Five days after infection, the sap from the nitrogen-deficient plants was only one-third as infective as that from the others, and after eight days the plants getting large dressings of nitrogen contained twelve times as much virus as the nitrogen-deficient plants although they were only three times larger. Between the fourth and twelfth days after inoculation, the virus content of the deficient plants increased only twenty times whereas the content of the others increased two-hundred times. WOODS and DU BUY (1941) suggest that in the nitrogen-deficient plants the virus is produced at least in part at the expense of the chlorophyll-proteins. They also suggest that in such plants the oxidase systems are deranged and that normal virus multiplication depends on these functioning properly. WOODS (1940) has also found that the multiplication of tobacco mosaic virus is inhibited if the infected leaves are treated with HCN, an effect which he also attributes to a poisoning of the oxidase systems.

SPENCER (1941*a* and *c*) claims that the activity of the virus, in addition to the quantity, is affected by the amount of nitrogen supplied to the host. When old infected plants were kept short of nitrogen, the amount of virus that could be isolated from them remained constant, but its infectivity per unit weight decreased by 40%. Similarly, when nitrogen was withheld from young plants ten days after they were inoculated, virus continued to be produced at a normal rate for a limited period, but weight for weight this was less infective than the virus produced in plants getting nitrogen continuously. SPENCER also describes an analogous variation in activity with the age of the lesion. He finds that virus in inoculated leaves continues to increase in quantity for as long as twenty days after inoculation, and that weight for weight the virus present twenty days after inoculation is four times as infective as that present after five days. The virus from the old lesions showed only one component when examined in the ultracentrifuge whereas that from the young lesions showed two. Unfortunately, SPENCER does not say which components gave the greater sedimentation constants and it is impossible to conclude whether or not these variations in infectivity could be explained simply on the basis of the aggregation and disaggregation of a basic virus particle. If the greatest activity per unit weight is not given by the preparation having the smallest sedimentation constant, then it would seem that there is an aggregate that has the optimum infectivity or that fully formed virus particles pass through a period in which their infectivity develops.

It has been shown earlier than the units of which the plant virus particles are composed are arranged with a perfect three-dimensional

regularity, and that the individual particles can be regarded as sub-microscopic crystals. PIRIE (1937) and STANLEY (1938) have both pointed out that in its simplest form virus reproduction may be analogous to crystal growth. It is well known that the introduction of a crystal "seed" of a substance into a saturated solution will result in the production of large numbers of similar crystals. Therefore, if the host cells are regarded as containing separately in solution all the constituent units of the virus particle, a virus particle entering the cell might behave as a crystal focus, cause the units to arrange themselves in an orderly manner alongside the units in the particle, and so to "crystallise" out as a single particle in the pattern of the infecting virus. Although this may provide a convenient picture for the final formation of a particle from its constituent units, it is almost certainly over-simplified, for it implies that the viruses have no specific activities and does nothing to explain the fact that different strains of the same virus, which presumably multiply in the same manner, produce widely different symptoms in the same host.

The suggestion has been made by STANLEY (1936), and more forcibly by NORTHROP (1938), that viruses are autocatalysts, which increase in susceptible cells merely by activating previously formed, inactive precursors. That the increase of viruses will at first be autocatalytic in the strict sense of the word, *i.e.*, proportional at any time to the amount already present, is almost certainly true, for the growth of nearly everything, from fires to bacteria, in an unrestricted medium is autocatalytic in this sense. It is doubtful, however, if viruses are merely the autocatalysts suggested by these workers. The analogy they use for the multiplication of viruses is the formation of proteolytic enzymes. NORTHROP has found that trypsin is not formed as such in the pancreas. From the pancreas he has isolated an inert protein, trypsinogen, and found that if a solution of this is inoculated with a trace of trypsin the inert protein is gradually transformed into active trypsin. In other words, the trypsin increases at the expense of trypsinogen. Similarly, from the gastric mucosa an inert protein has been isolated which is transformed by pepsin into active pepsin. This phenomenon is probably fairly general with intercellular proteolytic enzymes. Indeed, some such mechanism of production and removal from the cells in an inactive form would seem to be necessary if the contents of the cells are to escape hydrolysis by their own intercellular enzyme systems. On the other hand, there would seem to be no evolutionary significance in organisms producing inactive virus-precursors. There are, however, more powerful arguments than this rather teleological one against the view that viruses are simply autocatalysts.

In the first place, no sign of any virus-precursors has been found in healthy plants, and the inability of the viruses to multiply in extracts of susceptible organisms contrasts sharply with the ready increase of the proteolytic enzymes *in vitro*. The inactive precursors of the enzymes are in many ways similar to the enzymes themselves and readily change into them, but nothing at all similar to the viruses has been found in control, virus-free plants of susceptible species. It is true that some of these species contain proteins with large molecular weights, and hints have been made that these may be virus-precursors.

However, these have little in common with the viruses yet isolated except their large sizes. They are found equally in healthy and virus-infected plants, and differ widely from the viruses in their chemical and serological properties. Tobacco mosaic virus has been added to concentrated solutions of these normal plant proteins, but so far from there being any evidence of virus increase, the dilution of the virus in this manner caused a greater reduction in infectivity than an equal dilution in water or buffer solutions (BAWDEN and PIRIE 1938). Secondly, some plant viruses have such wide host ranges that it is necessary to assume that taxonomically unrelated plants contain the same precursors. Healthy plants of tobacco and phlox, for example, contain no demonstrable amounts of common antigenic materials, although each is susceptible to tobacco mosaic virus and, on the autocatalytic theory, should contain the virus-precursor. It is, of course, possible that healthy plants contain the precursor in quantities too small to be isolated or to give serological reactions. Then, if the precursor is necessary to the plant in small quantities, it is to be expected that as it is changed into virus more will be produced by the plant. In this way the production of large quantities of virus in plants containing no demonstrable amounts of precursors could be explained, but there are still further reasons why this seems improbable.

The tobacco plant is susceptible to so many different viruses and to so many strains of some individual viruses that it becomes a little difficult to imagine the healthy plants containing fully formed precursors for all of them. Also, it has been found with the proteolytic enzymes that the enzyme produced by the activation of the precursor is determined solely by the precursor and not by the enzyme used for activation. For instance, if trypsin is added to a solution of chymotrypsinogen, this is turned into chymotrypsin and not trypsin. Similarly, if swine pepsin is added to a solution of chicken pepsinogen, chicken pepsin and not swine pepsin is produced. This is quite different from the result obtained by infecting plants with viruses, where the resulting virus is determined solely by that used for infection. Many different strains of tobacco mosaic virus have been differentiated and studied. These, apart from occasional mutations, behave in a reasonably constant manner and can be transferred serially through many plants without undergoing any gross changes. For example, bulk cultures of aucuba mosaic virus remain recognisably aucuba mosaic virus after many years' passage through tobacco and tomato plants. On the autocatalytic theory such constancy could not be expected. Each plant would contain the precursors of all the many strains to which it is susceptible, so that when it was infected with aucuba mosaic virus all these should be transformed into their specific virus-strains. The result of such an infection, therefore, would not be a recognisable source of aucuba mosaic virus, but a mixture of roughly equal parts of all the strains of tobacco mosaic virus to which the plant is susceptible. In view of these gross differences between the behaviour of proteolytic enzymes and viruses, it would be premature to assume that they increase in similar ways until some positive evidence for the existence of virus-precursors has been found.

It is often assumed that viruses have no activities in their hosts

other than that of multiplication, the symptoms shown by infected plants being regarded merely as a result of "metabolic fatigue" produced by the continued change of normal cell constituents into virus. This view, however, ignores the fact that severity of symptoms is rarely a sure guide to the virus content of diseased plants. This is true even when plants are infected with related strains of the same virus. For example, the amount of virus in the sap of tobacco plants fully infected with potato virus "X" is not significantly different if the plants are infected with a strain causing severe ringspot or with one causing a barely susceptible mottle. Sometimes the virus content of plants infected with a strain causing severe symptoms may even be less than that of plants infected with a strain causing milder symptoms, the virus content of plants suffering from aucuba mosaic, for instance, is less than that of plants suffering from tobacco mosaic. There is every reason to believe that related strains multiply in the same manner. The fact that the presence of one strain prevents the multiplication of another can be explained simply only on the assumption that they multiply either at the same sites or by utilising the same materials, so that there is a competition between related strains. Then, if there is a maximum limit to the amount of any one type of virus that a plant can contain, as is indicated by the constancy of the virus content of plants grown in comparable conditions, a second strain would be unable to multiply in a plant already fully infected. From these facts it seems likely that the symptoms result from some effect other than virus multiplication. Possibly they are caused by the specific effects of active side groups of the virus particles, and the different strains cause their characteristic symptoms because they possess different side groups. The serological work which shows that different strains contain specific as well as common antigens is some support for the view that they contain such specific groups. On this view, therefore, it seems more reasonable to look upon a virus as a collection of active groups or of enzyme systems rather than as a single enzyme. Different strains of the same virus would differ in only a few of their active groups, and the mutation of one strain to another could be produced by the loss, or acquisition, or alteration of an active group, or even, perhaps, by the rearrangement of groups within the virus particle.

In normal cells protein synthesis proceeds along specific lines, each type of cell producing certain definite proteins and no others. Even in the same organism, different tissues frequently contain quite distinct proteins. This maintenance of characteristic individual proteins is attributed by BERGMANN and NIEMANN (1937) to the specificity of the intracellular proteinases. They have shown that papain in addition to its well known action of hydrolysing protein can at the same time synthesise peptide-like compounds. They suggest that in the cell the intracellular enzyme has at its disposal many protein fragments of different sizes and structure which it subjects to a series of transformations by synthesis, hydrolysis and replacement, so reconstructing one peptide bond after another, until there results a protein stable in the presence of the enzyme. If, as is probable, the intracellular proteinases are themselves proteins, there would occur the synthesis of one protein by another. BERGMANN and NIEMANN point out that such

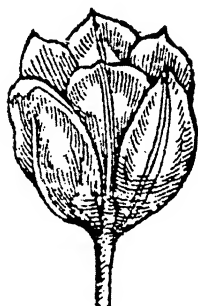
a proteinase with the ability to synthesise a replicate of itself instead of a different protein would behave exactly like a virus. Although there is no evidence that viruses have any proteinase activity, this view of virus reproduction seems at present the most probable. The most striking change in the protein content of plants is produced by tobacco mosaic virus. Although the total protein and nitrogen of tobacco plants do not seem to be greatly altered by infection, the quantity of soluble protein is definitely increased. This suggests that the virus multiplies largely at the expense of normally insoluble plant products. It is possible that the virus acts directly on these, liberating soluble fragments which are later built up into the virus particle. It is, however, equally possible that the virus itself has no direct hydrolytic effects on the cell contents, but acts indirectly by stimulating the normal plant enzymes, so that these produce the small units from which the virus is constructed. When plants are infected only leaves which are growing at the time actually show symptoms and come to have a high virus-content. This at first sight suggests a correlation between virus-multiplication and growth of the host. The correlation, however, is probably not real, and results from the fact that viruses normally enter mature leaves extremely slowly, for if such leaves are rubbed over their whole surface virus readily enters and multiplies.

In the theories outlined above it has been assumed that viruses are self-reproducing. Even this view, however, is not unanimously accepted. BORDET (1931) and others have suggested that viruses are abnormal products of their host's metabolism, and that when introduced into susceptible cells the viruses do not take an active part in their own multiplication, but merely modify the behaviour of the cells so that these produce virus instead of, or in addition to, their usual metabolic products. Thus it will be seen that although our knowledge on the nature of viruses and their properties *in vitro* has greatly increased in recent years, we are still almost entirely ignorant of their activities *in vivo*. Until much more is known of these activities it will be quite impossible to answer definitely the questions most frequently asked by biologists. Are viruses living? How do they multiply? How do they arise?

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